ANTIGEN PRESENTATION AND IMMUNE RESPONSE IN THE FEMALE RABBIT REPRODUCTIVE TRACT TO ANTIGENS DELIVERED BY RECOMBINANT MYXOMA VIRUSES

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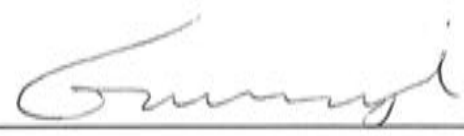
This thesis is my original work except where acknowledged.

Signature 

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COMMON-USED ABBREVIATIONS

APC	Antigen presenting cells
APS	Ammonium persulphate
BALT	Bronchus associated lymphoid tissues
BSA	Bovine serum albumin
cm	Centimetre
CTB	Cholera toxin B
DNA	Deoxyribonucleic acid
DC	Dendritic cells
DEPC	Diethylpryocarbonate
DTT	1,4-dithiothreitol
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
g	gram
GALT	Gut associated lymphoid tissues
HRP	Horse radish peroxidase
HA	Influenza virus haemoagglutinin
hCG	Human chorionic gonadotrophin
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IEL	Intra-epithelial lymphocytes
INF-gamma	Interferon gamma
IL-2	Interlukin-2
iu	International units
Kd	Kilo dolton
LP	Laminal propria
Mab	Monoclonal antibody

MAF	Mouse ascites fluids
mg	Milli gram
MHC	Major histocompatibility complex
MHC-I	MHC class I
MHC-II	MHC class II
min	Minute
ml	Milli litre
mM	Milli Mole
mRNA	Messenger RNA
MV	Myxoma virus
MV-HA	Recombinant myxoma virus expressing HA
MV-ZPB	Recombinant myxoma virus expressing zona pellucida protein B
NALT	Nasal associated lymphoid tissues
NK	Natural killer cells
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween
pfu	Plaque-forming units
pIgR	Polymeric Ig receptor
RC55	Rabbit zona pellucida protein B cDNA
RNA	Ribonucleic acid
RL-5	Rabbit CD4+ T cell line
SC	Secretory component
SDS	Sodium dodecyl sulfate
SIgA	Secretory IgA
SIgM	Secretory IgM
TEMED	Tetramethylethylenediamine
TNF-α	Tumor necrosis factor-alpha
TBS	Tris buffered saline
µl	Microlitre
µg	Microgram
ZP	Zona pellucida
ZPB	Rabbit zona pellucida protein B

ABSTRACT

The European rabbit (*Oryctolagus cuniculus*) is a major introduced vertebrate pest species in Australia, causing millions of dollars in economic losses each year and uncountable environmental damage. Current methods used to reduce the rabbit population are basically lethal and include shooting, trapping, poisoning, myxoma virus and rabbit haemorrhagic disease virus. These methods are expensive and have not been successful in reducing rabbit numbers to acceptable levels. An alternative, biological method of fertility control using a myxoma virus vectored immunocontraceptive vaccine, has been proposed by the Pest Animal Control CRC. For this proposal to be a success, an understanding of the profile of the immune response in the female rabbit reproductive system induced by antigens delivered in a recombinant myxoma virus and the selection of an effective immunocontraceptive antigen are crucial.

This project focused on understanding the immune response in the female rabbit to reproductive antigens delivered by the recombinant myxoma virus and assessing the immunocontraceptive potential of the reproductive antigens. To understand the basic elements of the immune system, essential immune cells such as T cells, B cells, and MHC class II+ cells and immunoglobulin distribution were examined in the oviduct, uterus, cervix and vagina both before and after ovulation. As in most reported species, large numbers of T cells and MHC class II+ cells were present in the reproductive tract of rabbits, especially in the cervix and vagina, whereas few B cells were present. KEN-5+ T cell and MHC class II+ cell numbers increased considerably in the uterus, but not in other sections of the tract, after ovulation. IgA and IgG were both present in the reproductive tract but there were few plasma cells. This suggested that these immunoglobulins were predominantly derived from serum.

The capacity of a viral vector to induce an immune response in the reproductive tract was directly investigated by infecting female rabbits with recombinant myxoma virus expressing the influenza haemagglutinating protein, a model strong antigen (MV-HA).

Intradermal inoculation of MV-HA induced high IgG titers to HA in serum and a low level of IgG to HA in the reproductive tract fluids but failed to induce an IgA response in either serum or reproductive tract fluids. Immunisation of female rabbits with MV-HA through intra-nasal or intra-vaginal mucosal routes also induced low levels of IgG to HA in reproductive tract fluids compared to serum and only a very low-level IgA antibody response.

In spite of the low antibody response in the reproductive tract, high titers of IgG to HA were always induced in serum following infections with MV-HA through any routes of immunisation. Although IgG antibody in reproductive tract fluids is thought to derive mainly from serum, the high-level of IgG antibody in serum did not transfer efficiently into the luminal fluids of the reproductive tract. In contrast, serum antibody freely entered the ovarian follicular fluids. The finding that the recombinant virus consistently induced a strong antibody response in serum and that the antibody freely entered the ovarian follicle indicates that recombinant myxoma virus could be used as a vector to deliver ovarian antigens for rabbit immunocontraception.

The study then investigated immunological and histological responses in the ovary to infection by a recombinant myxoma virus expressing an ovarian antigen, rabbit ZPB (MV-ZPB). Delivery of ZPB by MV-ZPB overcame self-tolerance to ZPB in female rabbits and induced a specific serum IgG response to this antigen. The IgG to ZPB specifically bound to the ZP of oocytes and this was associated with follicle disruption and other ovarian pathology.

It was concluded that if recombinant myxoma virus is to be used as a vector for rabbit immunocontraception, then it is more likely to be effective at inducing an immune response to an ovarian antigen than to an antigen located in the reproductive tract. The possible mechanisms underlying the immune response induced by recombinant myxoma virus, the implication of these results for developing a rabbit immunocontraceptive vaccine and future experiments are discussed in the final chapter.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Rabbit control in Australia: immunocontraception

The European rabbit (*Oryctolagus cuniculus*) is the major vertebrate pest species in Australia. It was introduced into Australia by European settlers in 1860 and then rapidly spread over continental Australia. It now causes hundreds of millions of dollars loss each year in agricultural production and untold environmental damage through land erosion and vegetation losses (Holland and Jackson, 1994).

The current methods used to control the rabbit population are basically lethal including trapping, poisoning, shooting and inducing viral diseases. However, these methods have disadvantages; some are costly, some are non-species specific (Holland and Jackson, 1994) and all meet with only partial success. In addition, they are considered inhumane by the public (Tyndale-Biscoe, 1994). As an alternative to these lethal methods, fertility control was suggested since it was believed to have significant advantages over lethal methods in controlling high fertility animals like the European rabbit (Bradley, 1994). To achieve this, the Pest Animal Control Cooperative Research Centre (PAC CRC) of Australia is exploring a new biological approach of fertility control by developing a viral vectored immunocontraceptive vaccine for rabbit population control. It is believed that this vaccine will be species-specific, cheap, and long lasting in rabbits because it is vectored by a live and rabbit specific virus, myxoma virus (Tyndale-Biscoe, 1994; Holland and Jackson, 1994).

In this chapter, the concept of viral vectored immunocontraception for rabbits will be introduced. Then the immune response and mechanism of blocking fertilisation in the

female reproductive tract will be reviewed. Next, this chapter will review and summarise the current antigen presentation strategies for inducing a strong antibody response in the female reproductive tract. And finally, as an alternative target for immunocontraception, immune responses in the ovary to ovarian antigens and effects of these on fertility will also be reviewed.

1.2 Viral vectored immunocontraception for rabbits

1.2.1 The concept and target

The proposal of viral vectored immunocontraception for rabbit population control was first suggested by Tyndale-Biscoe (1994) and also described by Holland and Jackson (1994). Figure 1-1 summarizes the concept. Genes encoding immuno-contraceptive antigens from rabbit gametes are isolated and cloned into the vector, myxoma virus, to make a viral vectored immunocontraceptive vaccine. When the recombinant virus is used to infect wild rabbits the virus would replicate in the infected

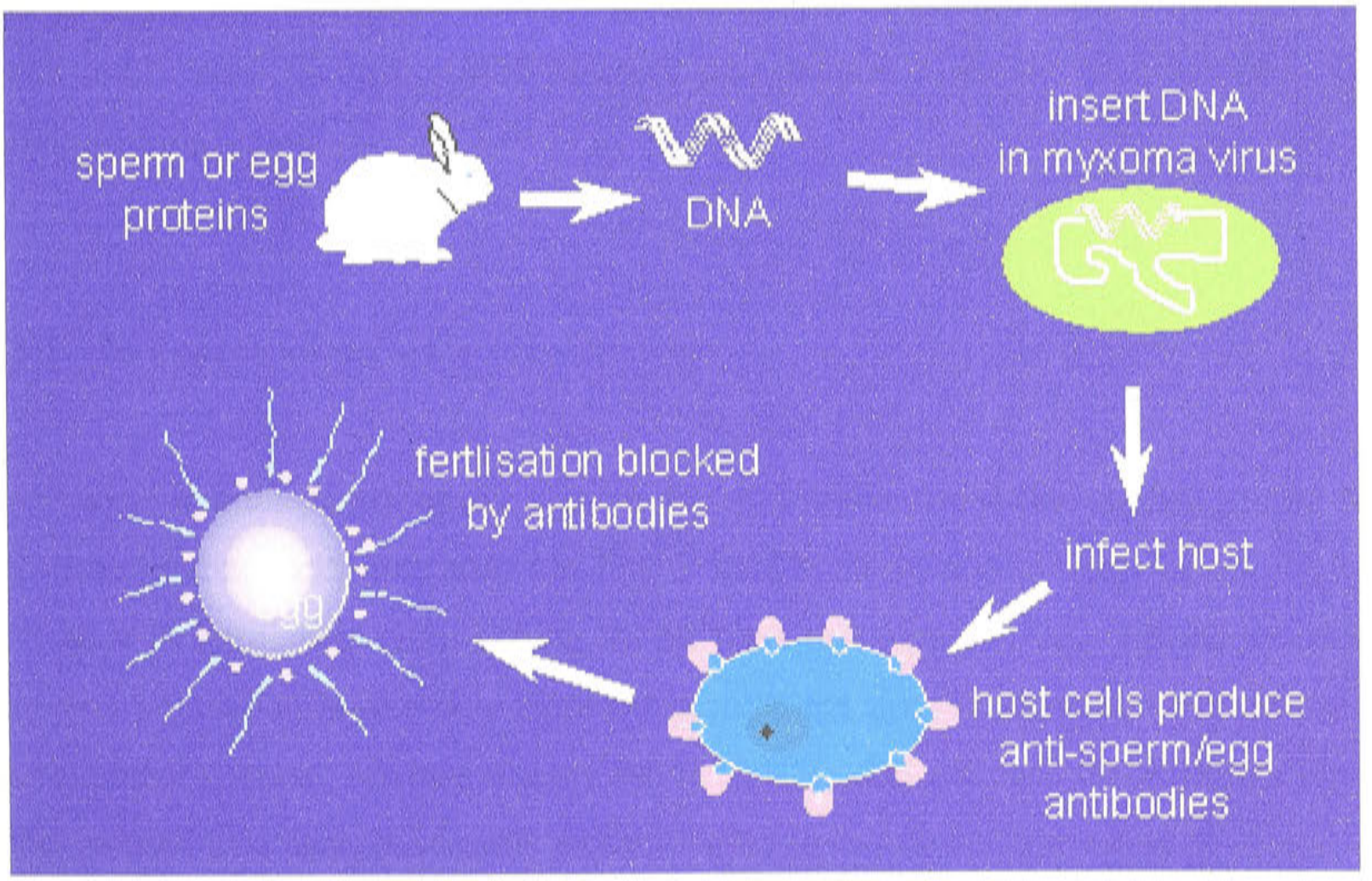


Figure 1- 1. The concept of viral vectored immunocontraception, proposed by PAC CRC (from PAC CRC Web site: www.pestanimal.org.au/).

cells and synthesise both viral and gamete antigen proteins (Holland and Jackson, 1994). The host immune system would recognise both viral and gamete antigens and mount both antibody and cell-mediated immune responses to them. Antibodies specific for the gamete antigen would enter the reproductive tract, bind to sperm or eggs and then block fertilisation.

According to this proposal, antigens from both male and female gametes could be vectored in myxoma virus and both male and female rabbits could be targeted for immunocontraception (Holland and Jackson, 1994). However, as fertilisation occurs in females there are some advantages in targeting them. Firstly, to the female, sperm antigens are foreign proteins and should generate an effective immune response. Secondly, oocyte or ovarian antigens could also be targeted. Although these are self-antigens, zona pellucida (ZP) proteins from heterogenous species may be used to overcome self-tolerance (Skinner *et al.* 1984; Jones *et al.* 1992). Finally, during fertilisation, both gametes are present in the female reproductive tract (Figure 1-2) where both can be targeted by an appropriate immune response. In particular, sperm-egg binding or fusion is considered to be species-specific (Holland and Jackson, 1994; Evans, 2000). Therefore targeting molecules involved in the interaction of sperm and egg increase the likelihood that a species-specific immunocontraceptive can be developed. This project will focus on female rabbits as targets for immunocontraception.

1.2.2 Myxoma virus as a viral vector

An ideal immunocontraceptive vaccine for wild rabbit population control should be self-disseminating, self-replicating and long lasting in the wild rabbit population. Above all it must be species-specific. During the 50 years that myxoma virus has persisted in the rabbit population in Australia there has been no evidence to show that the virus has infected other species (Tyndale-Biscoe, 1994). This is good evidence that myxoma virus could be used as a species-specific vector and led to its choice as a potential vector to deliver an immunocontraceptive vaccine (Tyndale-Biscoe, 1994; Holland and Jackson, 1994).

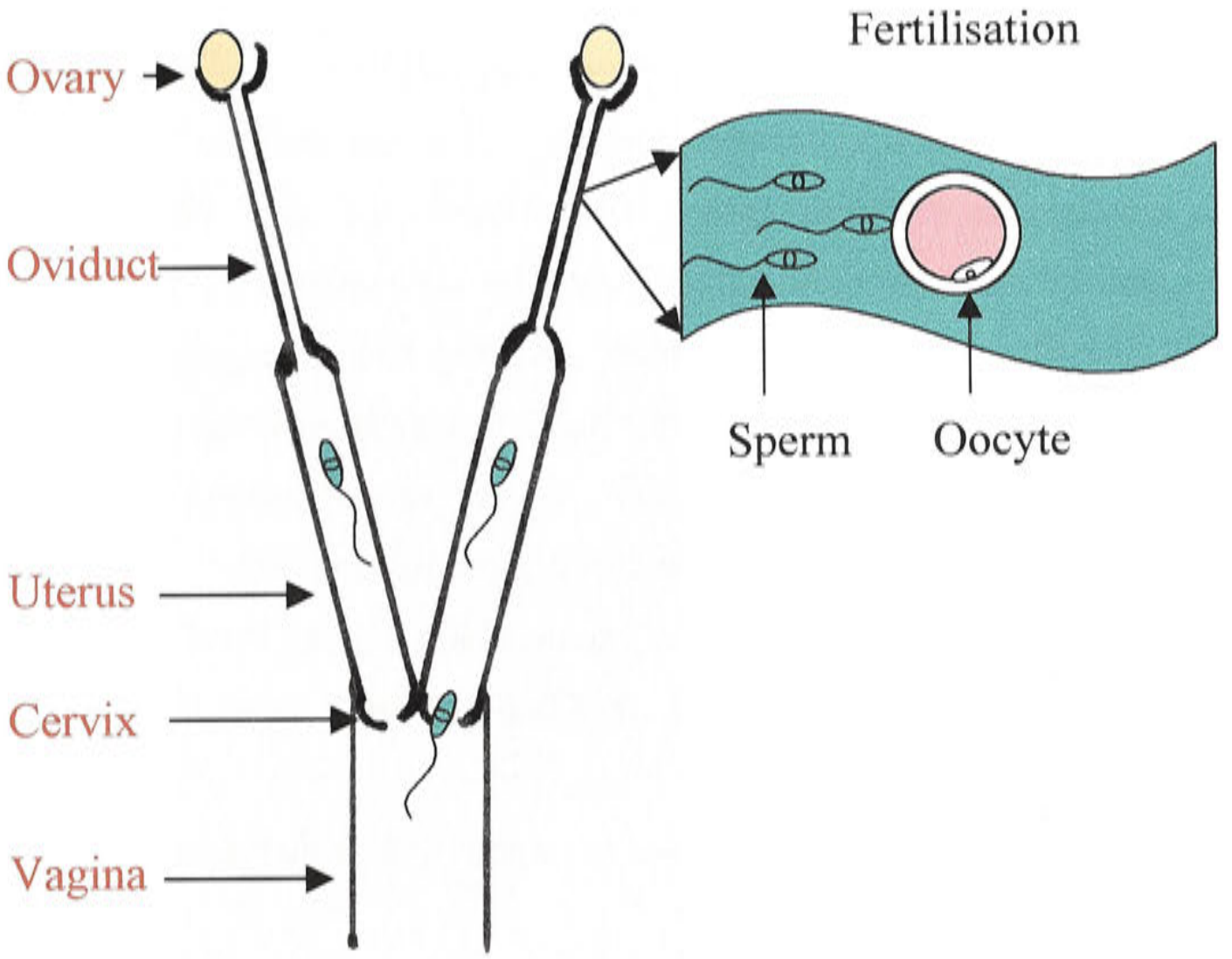


Figure 1- 2. Compartments of the female rabbit reproductive tract and fertilisation

The compartments of the female rabbit reproductive tract include the ovary, oviduct, uterus, cervix and vagina. In the rabbit, ovulation is induced by mating (Hammond and Marshall, 1925). The sperm deposited in the vagina swim through the cervices and uteri to the oviducts, where they bind to the oocytes, penetrate through the zona pellucida, fuse with the oocyte membrane and fertilise the egg. During this "long journey" in the female reproductive tract, sperm experience a series of preparations for fertilisation which include capacitation and the acrosome reaction which cause changes in their movement characteristics and surface membrane properties (Johnson and Everitt, 2000). The acrosome reaction allows the spermatozoa to release proteolytic enzymes from the acrosomal sac to facilitate penetration of the egg (Hunter, 1988).

1.2.2.1 The pathogenesis and disease course of myxomatosis

Myxoma virus is a poxvirus causing myxomatosis in the European rabbit. When it was first introduced into Australia in 1950 for rabbit population control, it killed >99% of infected rabbits (Fenner and Ratcliffe, 1965). However, the virus attenuated in the field and rabbits developed resistance to it and so the rabbit population recovered to a

considerable extent (Fenner and Ratcliffe, 1965). The pathogenic features of myxomatosis include extensive proliferative lesions at the primary sites of virus inoculation that are characterised by tissue degeneration and necrosis. By 7-10 days post-infection the virus has disseminated through the host and induces numerous secondary lesions, sometimes termed myxomas, that become visibly evident around the ears, eyelids, lips, nares, and genitalia. However, there is not sufficient histological damage in key organs to explain the high lethality of virulent strains of the virus. The accompanying dysfunction in host immunity can lead to supervening bacterial infections of the nasal and conjunctival mucosae and respiratory tract. Within 10-14 days rabbits infected with virulent strains experience dyspnea and die (Fenner and Ratcliffe, 1965, Fenner, 1994; Best and Kerr, 2000).

1.2.2.2 Viral replication and replication strategies

The virus is transmitted on the mouthparts of mosquitoes or other biting arthropods; it does not replicate in these vectors (Fenner, 1994). In the laboratory, intra-dermal inoculation of either Uriarra or SLS strain results initially in viral replication in dendritic-like MHC class II positive cells in the dermis (Best *et al.* 2000). About 24 hours later, the virus is found in T lymphocytes of the paracortical region in the draining lymph nodes of the inoculation site (Best *et al.* 2000). Between 4-10 days post-infection, the virus is found in peripheral blood mononuclear cells and in the spleen and lung (Best and Kerr, 2000). Clearance of the virus starts 10-15 days post-infection, depending on the strain of the virus and the resistance of the host rabbit (Best and Kerr, 2000). The main site of replication and transmission is in the epidermis.

Myxoma virus has developed strategies to evade, block, or modulate key effector molecules of innate or acquired arms of the host immune system. For example, it produces soluble homologues of TNF-α and INF-γ (Th1 cytokine) receptors that interfere with cytokine networks (McFadden *et al.* 1995; Nash *et al.* 1999). It is known that such cytokines play important roles in immune responses such as early T cell activation, dendritic cell maturation and antigen presentation (Roitt, 1997). Interfering with these cytokines would suppress the immune response against the infection. MV infection also down regulates MHC class I molecules on the surfaces of infected cells (Nash et al. 1999, Guerin *et al.* 2002) which will inhibit antigen presentation to

cytotoxic T-lymphocytes. Moreover, myxoma virus also specifically down-regulates the CD4 molecules on the surface of infected lymphocytes (Barry *et al.* 1995). This would result in the inhibition of recognition of antigen peptides presented by APC or B cells thus affecting activation of T cells and potentially the generation of cellular and humoral immune responses.

Myxoma virus also blocks migration of lymphocytes and monocytes to sites of active virus replication by inhibiting chemokine binding. The chemokines are small pro-inflammatory proteins which are important in activating and mobilising monocytes/macrophages, T-lymphocytes, natural killer cells, and dendritic cells to the sites of infection. The binding of the secreted viral protein M-T1 to CC-chemokines can prevent their engagement to their receptors (Nash *et al.* 1999; Lalani, *et al.* 1999). In addition, the viral T7 protein binds to and inhibits CC-, CXC- and C-type cytokines (Nash *et al.* 1999). Myxoma virus also produces molecules such as M-T2, M-T4, M-T5, and M11L that can modulate apoptosis of host lymphocytes (Zuniga, 2002). These molecules are essential for virulence, implicating lymphocytes as a key target cell for myxoma virus.

1.2.2.3 As a viral vector for delivering immunocontraceptive vaccine

Like other poxviruses, myxoma virus has been investigated as a potential antigen delivery vector, in this case for rabbits (Jackson and Bults, 1992). Insertions of the *E. coli* LacZ gene into either of two intergenic sites of the virus under the control of a poxvirus late promoter resulted in the expression of the foreign gene but the recombinant virus retained the virulence of the wild type virus (Jackson *et al.* 1996). Moreover, a recombinant myxoma virus expressing a model antigen, influenza haemagglutinin (HA), was shown to induce a strong HA antibody response in serum and a detectable level of IgG antibody in vaginal washings in infected rabbits (Kerr and Jackson, 1995). These results encouraged the view that recombinant myxoma virus could be used as a vector to deliver an immunocontraceptive vaccine.

The immune response in the reproductive tract has not been investigated in detail. It might be expected that the immuno-suppressive effects of myxoma virus would interfere with the immune response to antigens vectored by the virus. However, Kerr

and Jackson (1995) showed that after HA cDNA insertion the recombinant myxoma virus expressing HA was very attenuated. This attenuation of the recombinant virus may reduce the immunosuppressive effect and improve the immune response to viral or vectored antigens.

1.3 Sperm as a target for immunocontraception

1.3.1 The role of antibody in blocking fertilisation

An advantage of using a sperm antigen for immunocontraception is that both male and female rabbits can be targeted simultaneously (Holland and Jackson, 1994). As described above, spermatozoa and some seminal proteins are antigenically foreign to females and so are more likely to generate an effective immune response in females than a female self-antigen such as an oocyte protein. Therefore, sperm antigens were considered as the first choice for an immunocontraceptive antigen.

Early data from rabbits demonstrated that an antibody response to rabbit semen (Menge and Lieberman, 1974) or sperm-specific lactate dehydrogenase (Goldberg, 1973) was induced in females following intra-dermal or intra-muscular immunisations and a block in fertilisation was achieved. Antibodies to sperm antigens of both IgG and IgA classes in uterine fluids were shown to block fertilisation *in vitro* (Menge and Lieberman, 1974). But serum antibody levels to sperm-specific lactate dehydrogenase did not correlate with the significantly low pregnancy rate (Goldberg, 1973). It was concluded that fertilisation can be blocked by immunisation with sperm or sperm components and that antibodies in the reproductive tract fluids play a principal role in this.

Spermatozoa are deposited in the female reproductive tract after mating and travel from the vagina via the cervix and the uterus to the oviduct where they bind to and fertilise the eggs (Figure 1-2). Therefore for immunocontraception to be most effective, local antibodies are ideally required throughout the reproductive tract to attack spermatozoa during their "long journey". Antibodies to sperm antigens in the female reproductive tract could agglutinate or immobilise the sperm (Metz, 1972).

Antibody binding to sperm antigens could also activate complement and lead to local inflammatory responses that might destroy or damage the spermatozoa (Goldberg, 1973). The fewer spermatozoa that arrive at the oviduct, the lower the chance of the egg being fertilised. More importantly, specific antibodies to sperm-oocyte binding molecules can also block spermatozoa attaching to oocytes and penetrating through the oocyte zona pellucida and so block fertilisation in a specific manner. A study in rabbits showed that the Fab fragment of antibody to a rabbit sperm antigen (termed RSA) could bind to the post-acrosomal region of the sperm and inhibit sperm-zona binding and penetration both *in vitro* and *in vivo* (O'Rand *et al.* 1988). Several monoclonal antibodies to rabbit sperm protein epitopes were also established and shown to be able to block fertilisation *in vitro* (Holland and Jackson, 1994). These data suggest that antibody to specific epitopes on sperm can effectively block fertilisation. In addition, antibodies to sperm penetrating enzymes such as hyaluronidase were shown to inhibit fertilisation *in vitro* and the Fab fragment of specific IgG could effectively do this, suggesting that agglutination and immobilisation of the sperm might not be an essential prerequisite for infertility of female rabbits (Dunbar *et al.* 1976). Another study in rabbits also showed that active immunisation of female rabbits with rabbit sperm antigen GA-1 resulted in reduction of fertility although the specific antibody to this antigen had no effect on sperm agglutination and immobilisation (Naz *et al.* 1986). These data indicated that antibody to sperm antigens can block fertilisation by preventing sperm-egg interactions.

Obviously, if an immunocontraceptive vaccine based on spermatozoon antigens expressed in myxoma viruses is to be successful two things are necessary. Firstly, an immunocontraceptive sperm antigen needs to be identified (Holland and Jackson, 1994; Holland, 1997), and secondly a good understanding of whether the recombinant myxoma virus can induce a satisfactory antibody response in the female rabbit reproductive tract is critical. This induction of an antibody response in the female rabbit reproductive tract to an antigen delivered by recombinant myxoma virus is the major topic of this study.

1.3.2 Induction of an effective antibody response in the reproductive tract

Data from early studies in rabbits showed that anti-sperm antibodies and infertility could be achieved following immunisations with sperm antigens (section 1.3.1). However, to achieve these, Freund's complete adjuvant and several boosts were usually needed for the immunisation regimes and even then, a consistent and effective antibody response in the female reproductive tract was not obtained. For example, female rabbits immunised by intra-muscular injection with complete semen produced no antibodies in the reproductive tract fluids though antibodies were detected in serum (Edward, 1960). Similar results were also obtained following immunisation with yeast alcohol dehydrogenase (Shapiro *et al.* 1971) where only 4% of the serum antibody level to this antigen was present in oviduct fluids (Oliphant *et al.* 1977). Furthermore, rabbits immunised with sperm antigen LDH-C4 had only 3-4% of the serum antibody titer in oviductal fluids (Kille and Goldberg, 1979). Freund's adjuvant and boosting were used in these studies but were not effective in consistently inducing a strong immune response in the female reproductive tract.

Recent studies showed that female rabbits immunised with HIV-1 C4/V3 peptide supplemented with cholera toxin elicited specific IgG in serum and specific secretory IgA (sIgA) in vaginal secretions when the immunisation was delivered via Thiry-Vella ileal loops (Winchell *et al.* 1997). Similarly, specific sIgA response to the peptide in vaginal secretions was increased when using a DNA vaccine delivered by gene gun into Peyer's patches (Winchell *et al.* 1998). In contrast, in another study in rabbits it was shown that immunization through Peyer's patches with rabbit sperm antigen PH30 and cholera toxin B was unsuccessful in inducing a specific IgA response in vaginal washings though a strong specific IgG response was detected in serum (Hardy *et al.* 1997). These results indicate that delivery of the antigens into a mucosal compartment may be important in eliciting a good immune response at a distal mucosal site. However, the difference between a DNA vaccine or PH30 antigen immunisation into Peyer's patches in terms of the IgA response may reflect the continuing expression and longer persistence of the DNA plasmid compared with the PH30 protein.

Immunisation routes play an important role in inducing a mucosal immune response. Local, but not systemic, immunisation of female rabbits with a model antigen, horseradish peroxidase (HRP), induced a detectable IgA response in reproductive tract fluids (McAnulty and Morton, 1978). Moreover, it was shown that intranasal immunisation of rabbits with HIV-1 C4/V3 peptide elicited a better serum IgG and vaginal sIgA response than immunisations via systemic or other mucosal routes (Zinckgraf *et al.* 1999). In the mouse, the initial route of immunisation was important in determining the type of immune response (Kelly *et al.* 1996). For example, mice immunised via the intra-nasal, oral, and vaginal routes with live MoPn (mouse pneumonitis biovar of Chlamydia trachomatis) developed a dominant Th1-type cytokine profile with the ratio of gamma interferon-secreting cells to IL-4-secreting cells greater than 10 in lymph nodes and spleen. In contrast, mice subcutaneously injected with MoPn produced a Th2-type profile with a gamma interferon /IL-4 ratio of only 0.7. These mice also had significantly higher anti-MoPn IgG1 serum titres, confirming a Th2-type cytokine profile (Kelly *et al.* 1996). Furthermore, a previous study showed that dendritic cells (DC) within different tissues have individual microenvironments and induce different cytokines. For example, DC in spleen mainly induce Th1 cytokines while DC in Peyer's patches predominantly induce Th2 cytokines (Everson *et al.* 1996). These data collectively suggest that the use of appropriate antigen presentation strategies such as using DNA plasmid vectors or viruses to deliver antigens and mucosal immunisation routes are likely to be very important in induction of an effective antibody response in the female reproductive tract.

The use of recombinant virus to deliver antigen results in replication of the antigen and penetration through mucosal surfaces when administered at mucosal sites, both of which are considered to be important for inducing a mucosal antibody response (Parr and Parr, 1994; 1996). In the mouse, recombinant adenovirus was able to deliver a herpes simplex virus type 1 (HSV-1) antigen and induce effective mucosal antibody (Gallichan *et al.* 1993, Gallichan and Rosenthal, 1995) and cell-mediated immune responses (Gallichan and Rosenthal, 1996) in the female reproductive tract. But in rabbits, it was unknown whether a recombinant myxoma virus could achieve this. However, it is necessary for the virus to do this if it is to be used to deliver immunocontraceptive antigens that induce immune responses within the reproductive tract sufficient to block fertilisation. Understanding this is a major aim of this project.

1.4 Antibody and its transfer in the female reproductive tract

1.4.1 IgG antibody in the tract and passive transfer

Although antibodies of IgG, IgA and IgM classes are all present, IgG is the dominant antibody class in the female reproductive tract fluids for most species (Parr and Parr, 1996). This is different to other mucosal sites such as the intestinal and respiratory tract where IgA is the dominant antibody class in the luminal secretions (Brandtzaeg, 1995, 1997). In rabbits, early data showed that IgG was the dominant Ig in the uterine and vaginal fluids with IgA being only detected in some instances in the vaginal fluids (Symons and Herbert, 1971), though IgG, IgA and IgM were all present in the oviductal fluids (Oliphant *et al.* 1977). IgG was also the only antibody class detected in reproductive tract fluids of female rabbits intramuscularly immunised with a model antigen, HRP (McAnulty and Morton, 1978). Similar results were reported in rabbits following immunisation with sperm antigen (Kille and Goldberg, 1979; Menge and Lieberman, 1974) or infection with a recombinant myxoma virus expressing influenza HA (Kerr and Jackson, 1995). In humans (Mestecky and Fultz, 1999) and mice (Su *et al.* 1995), IgG was also demonstrated to be the dominant antibody class in the female reproductive tract fluids.

The IgG present in the reproductive tract fluid is considered to be mainly derived from serum and IgG can passively cross the epithelium of the reproductive tract into the luminal fluids by means of intercellular diffusion or intra-cellular vascular transport across epithelial cells (Parr and Parr, 1994, 1996). However, in most cases, epithelium acts as a boundary between tissue and lumen so that the concentration of Ig in luminal fluids is much lower than that in the tissue or circulation. Compared with serum, female rabbit reproductive tract fluids contained only 20% of the IgA and less than 10% of the IgG and IgM (Oliphant *et al.* 1977). This implies that high levels of serum IgG are likely to be important for achieving a level of IgG antibody in reproductive tract fluids that would be satisfactory for achieving infertility. Recombinant myxoma virus expressing influenza HA induced a strong antibody response to HA in serum (Kerr and Jackson, 1995) and this could provide a source for transfer of antibody into the reproductive tract fluids.

Although serum transudation is the main source of IgG present in the reproductive tract, local IgG antibody production may also be possible in the murine reproductive tract. This is suggested by the finding of many IgG plasma cells in the murine vagina after infection with HSV-2 (Parr and Parr, 1997). In addition, IgG plasma cells were also reported in the mucosa of the rabbit vagina (Symons and Herbert, 1971). These plasma cells may contribute to local IgG production and provide a source of IgG for transfer into the reproductive tract fluids. Thus, IgG present in the lumens of some compartments of the reproductive tract may derive from both serum and local production.

1.4.2 Secretory IgA and mucosal immunity

The female reproductive tract is considered to be a part of the common mucosal immune system (Parr and Parr, 1994, 1996; Brandtzaeg, 1997). It is characteristic of mucosal immunity that secretory IgA and IgM (sIgM) are present in mucosal secretions. The general function of secretory immunoglobulins is to perform immune exclusion, which excludes pathogens or other toxic materials from the body and perhaps returns antigens, which have penetrated epithelial cells, to the lumen (Parr and Parr, 1996; Brandtzaeg, 1997). Adequate levels of IgG, sIgA, and sIgM in the reproductive tract are essential for protecting the tract from infection; low levels of these Igs in the uterus have been linked to the occurrence of endometritis (Aknazarov, 1988). Besides the general function of secretory Ig in local immunity, sIgA antibody could also neutralise antigens such as viruses in the epithelial cells and in the lamina propria (Kaetzel *et al.* 1991; Mazanec *et al.* 1992, 1993). Because sIgM concentration in reproductive tract fluids is usually much lower than sIgA and is more easily degraded in mucosal secretions than sIgA (Brandtzaeg, 1997), sIgA antibody is thought to provide a major and lasting immunity in the reproductive tract. In addition, it was suggested that the sIgA antibody could be more effective than IgG antibody in neutralising antigens because it has more antigen binding sites and so can form a bigger antigen-antibody complex to be cleared (Parr and Parr, 1996). Therefore, from this point of view, an ideal antibody response for immunocontraception would include both IgA and IgG in the female reproductive tract. However, it is not clear if sIgA would be more effective than IgG in blocking fertilisation.

Rather than being derived from serum, sIgA present in the luminal fluids is believed to be mainly derived from local production by IgA plasma cells in the reproductive tract tissue (Parr and Parr, 1996; Brandtzaeg, 1997). This is supported by the presence of a greater number of IgA plasma cells compared to other plasma cells in the female reproductive tract in several species (Rachman *et al.* 1983; Parr and Parr, 1996; Brandtzaeg, 1997). In mice, it was shown that numerous IgA plasma cells were present in the uterus at pro-oestrus and oestrus with very few IgG plasma cells found at any stage of the oestrous cycle. Moreover, IgG was present throughout the stroma, suggesting that IgA may be sourced mainly from local production while IgG may be derived mainly from serum (Rachman *et al.* 1983). IgA and IgG plasma cells were present in the uterine glands and in the gland lumen in the horns and body of the mouse uterus with the ratio of cells containing IgA to cells containing IgG being 3 or 4:1 at all stages (Parr and Parr, 1985). Predominant IgA with less frequent IgG plasma cells were also reported in the human fallopian tube (Kutteh *et al.* 1990). In the sow, although all three types of immunoglobulin secreting cells were distributed in all regions of the reproductive tract, IgA secreting cells were dominant (Hussein *et al.* 1983). Because of the dominant distribution of IgA plasma cells in the reproductive tract and their production of dimeric IgA, these cells are believed to be the prime source of sIgA present in the luminal fluids. For the rabbit, limited information showed that there were some IgA plasma cells in the endometrium whereas IgG and IgM positive cells were not found (Otsuki *et al.* 1990), suggesting that local IgA production could occur. However, information regarding the distribution of IgA plasma cells in other regions of the female rabbit reproductive tract and IgA production or transport in the tract is not available.

Although, as described above, there is good evidence for local production of IgA in the reproductive tract, there is also evidence that transudation from serum may also be important. For example, in rats, IgA plasma cells were not found in any regions of the female reproductive tract at any stage of the oestrous cycle, suggesting that the IgA present in the rat reproductive tract fluids must mainly be derived from serum (Parr and Parr, 1989) and in human, monomeric or dimeric IgA in serum also provides a source for IgA in cervical secretions (Parr and Parr, 1996; Mestecky and Fultz, 1999). In rabbits, it is not clear whether serum IgA would contribute to IgA present in reproductive tract fluids.

Unlike IgG, the transfer of dimeric IgA produced by local plasma cells into luminal fluids is a selective process, which requires the polymeric Ig receptor (pIgR) on the basal membrane of epithelial cells. The detailed transfer process is shown in Figure 1-3. After transfer across the epithelial cells the dimeric IgA contains an extra secretory component (SC) from pIgR (Figure 1-3), which protects the antibody from cleavage by proteolytic enzymes in secretions. The transfer of dimeric IgA derived from serum into luminal fluids is conducted by the same process as local dimeric IgA but serum monomeric IgA is transferred by the same passive mechanisms as IgG (Parr and Parr, 1996). The transfer of IgM across the epithelial cells is generally thought to also need the pIgR (Brandtzaeg, 1997). However, pIgR may be not required for rabbit IgM transfer because this receptor may not bind to IgM (Underdown *et al.* 1992).

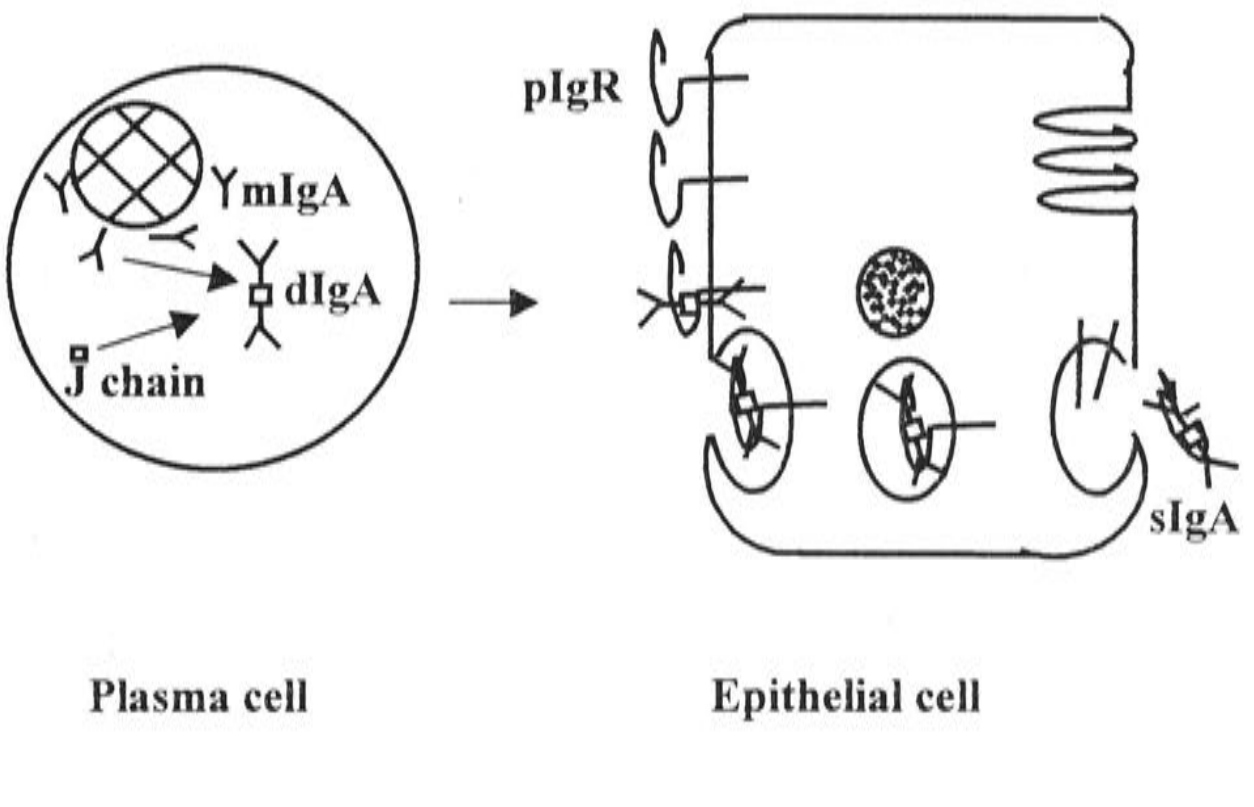


Figure 1- 3. Transfer of dimeric IgA through the pIgR on epithelial cells

Locally produced dimeric IgA, dIgA, consists of two monomeric IgA (mIgA) molecules linked together by a J chain. The dIgA binds to the polyimmunoglobulin receptor (pIgR) on the basal membrane of epithelial cells. The antibody/receptor complexes are taken into the epithelial cells by endocytosis. The dimeric IgA is cleaved from the receptor in the vesicles of the epithelial cell after which secretory IgA (sIgA: secretory component, SC together with dIgA) is released into luminal fluids by exocytosis. (Adapted from McGhee *et al.* 1999).

Clearly, to be able to induce an effective IgA response in the reproductive tract, an antigen presentation strategy that could induce more IgA plasma cells to be present in the tract is important. A previous study showed that mucosal immunisation resulted in

99% of specific antibody-secreting cells (ASC) bearing the homing receptor α4β7 integrin which targets the cells to mucosal effector sites whereas systemic immunisation only induced 58% ASC cells bearing the same receptor (Kentale *et al.* 1997). This suggests that immunisation through mucosal sites induces more ASC that home back to mucosal sites. Furthermore, it is now generally agreed that antigen primed B cells mostly home back to where they were primed (Brandtzaeg, 1997). This also suggests that mucosal immunisation needs to be considered to induce an effective IgA plasma cell response that homes to the reproductive tract.

1.4.3 Rabbit IgA isotypes

One important difference between rabbit IgA and IgA of other species is the rabbit has the potential to produce as many as 13 IgA isotypes, Cα1-13 (Spieker-Polet *et al.* 1993). Most of these IgA isotypes are expressed although some (Cα4, Cα5, Cα6, Cα9, Cα10, Cα12, and Cα13) are expressed at high levels and others (Cα1, Cα2, Cα7, and Cα11) are expressed at low levels. Cα3 and Cα8 are not expressed because of mutations in the promoters (Spieker-Polet *et al.* 2002). In addition, it was shown in rabbits that different tissues express different IgA isotypes. For instance, Cα1, 6, 9, 10 and 12 are expressed in small intestine, mammary gland, salivary gland and appendix whereas Cα4 is only expressed in lung and spleen (Spieker-Polet *et al.* 1993). It is unknown how many isotypes are expressed in the reproductive tract of female rabbits. However, these findings suggest that induction of an IgA antibody response in the rabbit reproductive tract may be complex and depend upon the site of antigen presentation and the microenvironment in which the B cells differentiate.

1.4.4 Diversification of the antibody repertoire

There are also some characteristic features of rabbits compared to the standard human and mouse models regarding the development of the antibody repertoire. Somatic diversification of Ig genes is an important step in this process. In mice and humans, it occurs as a part of the T-cell dependent immune response in peripheral

lymphoid tissues in response to antigen stimulation (Knight and Winstead, 1997). In rabbits and some other species such as chicken, sheep, and cattle, somatic diversification is responsible for the primary antibody repertoire and it occurs in gut-associated lymphoid tissue (GALT, Vajdy *et al.* 1998). In chicken and sheep, diversification occurs entirely during fetal development and is independent of exogenous antigen (Knight and Winstead, 1997). But in rabbits, somatic diversification occurs post-natally and nearly all Ig VDJ genes are somatically diversified by about 2 months of age (Vajdy *et al.* 1998). An interaction with specific but currently unidentified intestinal microflora is essential for the diversification (Lanning, et al. 2000). In addition, the rabbit is one of a few vertebrates that make a limited use of combinatorial joining of multiple VH, DH, and JH gene segments during Ig heavy chain gene rearrangements. The first 3' VH gene segment, VH1, is utilised at a rate of 80-90% though there are 100 genes available (Knight, 1992). Thus, the rabbit has a very limited capacity to use combinatorial joining in generating a diverse primary repertoire of antibody (reviewed in Knight and Crane, 1994). Rather than using combinatorial joining, the rabbit uses somatic gene conversion to generate antibody diversity. This character may influence the antibody response in rabbits and thus the antibody response in the reproductive tract to antigens delivered by recombinant myxoma viruses.

1.5 Antigen presentation and induction of a mucosal antibody response in the female reproductive tract

1.5.1 Antigen presentation via common mucosal inductive sites

The female reproductive tract is considered to be a compartment of the common mucosal immune system. After immunisation through a common mucosal inductive site, lymphocytes including IgA plasma cells are expected to home back to the effector site including the female reproductive tract (McGhee *et al.* 1999). The general concept of the common mucosal immune system is summarized in Figure 1-4. In this system, the antigen presenting cells (APC), M cells, in the inductive site play an important role in transporting antigen and presenting antigen peptides to T cells in the local lymphoid tissues. B cells can also be activated by antigens in the local environment or by intact

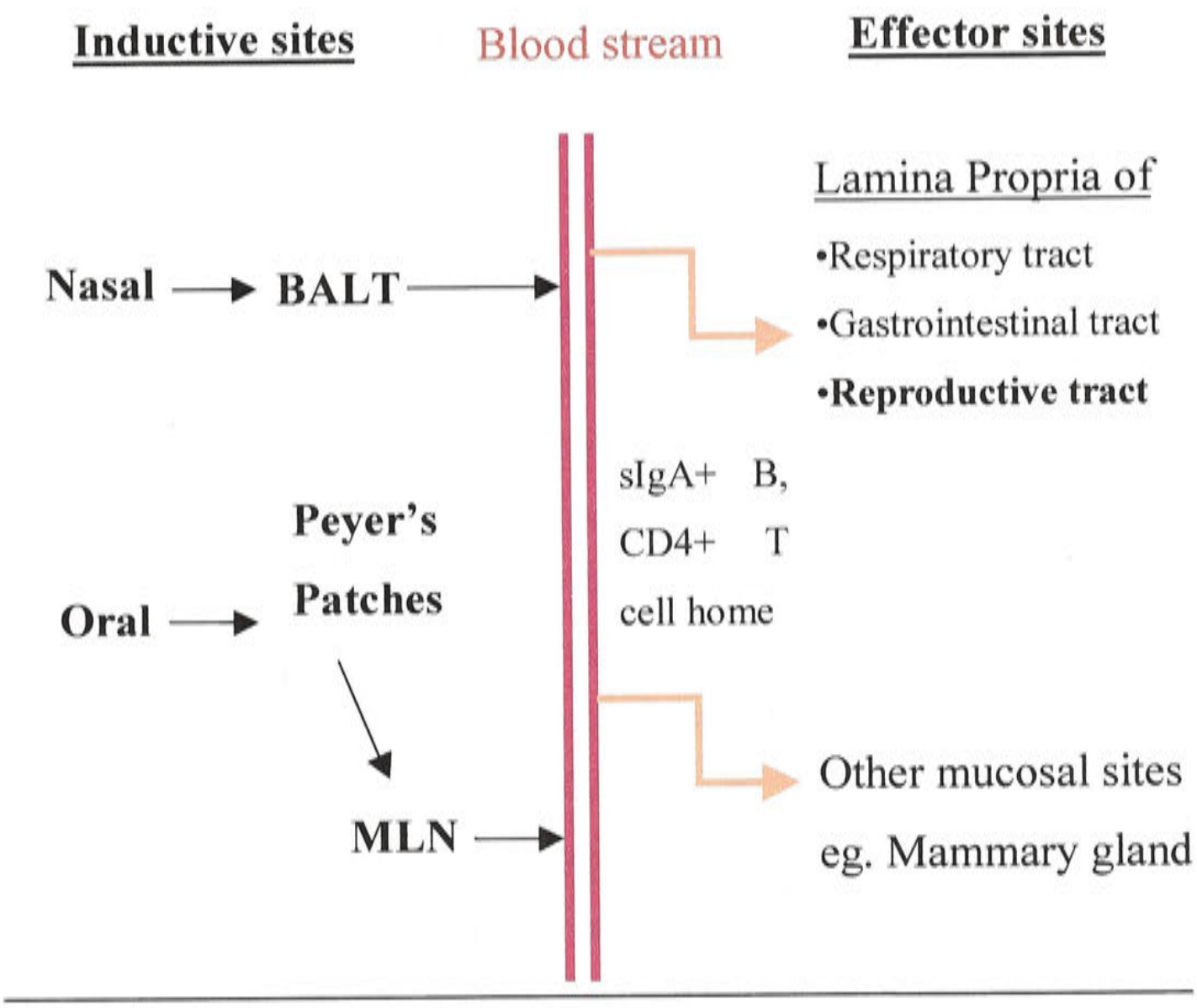


Figure 1- 4. Inductive and effector sites of common mucosal immune system

Antigens administrated through nasal or oral routes are exposed to common mucosal inductive sites such as BALT (bronchus associated lymphoid tissues) and Peyer's patches (also GALT, gut associated lymphoid tissues). The antigens are taken up and transported by APC (M cells) to local lymphocytes in these lymphoid tissues (McGhee *et al.* 1999). The B and T lymphocytes in the inductive sites that are reactive with the antigen can then be activated and differentiate. In the respiratory tract the antigen-primed lymphocytes may directly move into the blood stream. In the intestine, the antigen primed T or B lymphocytes migrate to local lymph nodes (MLN, mesenteric lymph nodes) for further differentiation, which may involve expression of homing receptors, and then enter the blood stream. Some of the antigen specific sIgA bearing cells or CD4+ T cells would home back to effector sites such as the lamina propria of the gastrointestinal, respiratory and reproductive tract. The sIgA+ cells would then contribute to local IgA production at this site.

antigens on the cell membrane of APC together with co-stimulation signals (eg. cytokines or cell-cell interaction) from activated CD4+ T helper cells (Roitt, 1997). After activation, the primed B cells will express homing receptors such as $\alpha 4\beta 7$ during

their differentiation, which will lead these cells to home back to mucosal effector sites including the female reproductive tract (McGhee *et al.* 1999). These plasma cells would produce specific IgA, which would then be transferred into the lumen as described in section 1.4.2.

In the rabbit, immunisation through both nasal and intestinal inductive sites is capable of inducing a mucosal antibody response in the female reproductive tract (section 1.3.2). Compared with the intestinal route, the nasal route of immunisation was more effective in inducing a remote specific IgA response in the female reproductive tract of rabbits (Zinckgraf *et al.* 1999, section 1.3.2). In the mouse, intra-nasal immunisation was also more efficient than other routes in inducing mucosal immune responses in the female reproductive tract (Gallichan and Rosenthal, 1995; de Haan *et al.* 1995; Johansson *et al.* 1998; Xiang *et al.* 1999). For example, intranasal immunization with recombinant adenovirus expressing HSV-1 glycoprotein B was superior to the intra-vaginal route in inducing specific IgA responses in serum and vaginal secretions (Gallichan *et al.* 1993; Gallichan and Rosenthal, 1995). Specific antibody-secreting cells were also shown to home back to the reproductive tract after intra-nasal immunization with the same recombinant virus (Gallichan and Rosenthal, 1998). At the start of this study, it was unknown if mucosal immunisations of rabbits with a recombinant virus could induce an IgA antibody response in the female reproductive tract.

1.5.2 Local antigen presentation in the female reproductive tract

The ability of immune cells in the female reproductive tract to present antigen and mount a local immune response is important for developing immunocontraceptive vaccines and vaccines against sexually transmitted diseases. That this can occur is demonstrated by the achievement of local immunity in the reproductive tract to viral infection of herpes simplex virus-2 (HSV-2, McDermott *et al.* 1989; Parr and Parr, 1997). However, unlike other mucosal sites such as the intestine and respiratory tract, the female reproductive tract lacks antigen processing cells (M cells) and organised lymphoid tissues such as lymph nodes and lymphoid nodules (Parr and Parr 1996). This

suggests that antigen presentation in the reproductive tract, if it occurs, would be different to other mucosal sites.

APC such as macrophages, dendritic cells and Langerhans' cells are present in the female reproductive tract of rats (Kaushic *et al.* 1998), mice (Parr and Parr, 1991) and humans (Morris *et al.* 1985; Givan *et al.* 1997; White *et al.* 1997) based on immunohistochemical or flow cytometric analysis with monoclonal antibodies to specific markers for these cells. MHC class II positive cells were also reported in the female reproductive tract of human (Ljunggren and Anderson, 1998; Johansson *et al.* 1999) and rats (Kaushic *et al.* 1998). Based on the observation that many APC are present in the lower reproductive tract but the reproductive tract lacks organised lymphoid tissues, the pathway of antigen presentation and immune response in the female reproductive tract is probably through the draining lymph nodes (Parr and Parr, 1991; McGhee *et al.* 1994). According to this pathway, local APC are responsible for picking up and transporting antigens present at the mucosal surface of the vagina to the draining lymph nodes of the genital tract. The APC will also process the antigen and present antigenic peptides on MHC class II molecules to CD4+ T lymphocytes in the draining lymph nodes to activate these T cells. B cells in the draining lymph nodes could also be activated by intact antigens transported by these APC together with the signals from activated T cells as described in 1.5.1 or by direct interaction with antigens on dendritic cells (Wykes, et al. 1998). The antigen-specific lymphocytes, including committed specific IgA producing B cells, would move into the blood stream and home back to the reproductive tract and other mucosal sites. These specific IgA producing cells would then produce IgA that would be transferred into the lumen as previously described in section 1.4.2.

There is some evidence supporting this pathway. Intra-vaginal vaccination of mice with an attenuated strain of HSV-2 rapidly induced immunity to a lethal intra-vaginal challenge with wild type HSV-2. This resistance was transferable to syngeneic mice with genital lymph node cells but not with cells from other lymphoid tissues (McDermott *et al.* 1989). HSV-2-activated T cells in the genital lymph node were essential for resistance to genital infection by HSV-2 (McDermott *et al.* 1989). Similarly, macaques immunised orally followed by vaginally or vaginally followed by orally with a recombinant particulate simian immunodeficiency virus (SIV) antigen

linked to cholera toxin B elicited a specific CD4+ T cell response to the antigen in the genital lymph nodes and the spleen but not in unrelated lymph nodes. B cells, together with appropriate help from CD4+ T cells and macrophages from the genital lymph nodes produced specific IgA and to a lesser extent, IgG antibodies. However, intra-muscular immunisation induced splenic but not genital lymph node cells and stimulated CD4+ T cell proliferation and predominantly B cell IgG antibody synthesis (Lehner *et al.* 1995). Furthermore, in the mouse, ASC to cholera toxin B or cholera toxin B conjugated with human gamma globulin were found in the genital lymph node following intra-vaginal immunisations (Johansson *et al.* 1998). These data support the model that specific T cell and B cell responses can be induced in the local draining lymph nodes of the reproductive tract following intra-vaginal immunisation.

Various studies have demonstrated that local immunisation in the female reproductive tract was an effective way to stimulate a local IgA response. In the rabbit, a detectable specific IgA response was induced in reproductive tract secretions following intra-vaginal or intra-uterine immunization followed by intra-vaginally boosting with HRP (McAnulty and Motton, 1978). Similarly, macaques immunised vaginally and boosted orally with recombinant SIV antigen produced specific IgA and IgG antibodies in vaginal fluid and in serum (Lehner *et al.* 1992). Intra-vaginal immunization was also an effective way to induce local mucosal immune responses in the female genital tract of mice (Johansson *et al.* 1998; Kato *et al.* 2000), rats (Menge *et al.* 1993), and humans (Kozlowski *et al.* 1997). A neutralising IgA and IgG antibody response to HIV-1 envelope protein was also elicited in mouse vaginal secretions after intra-vaginal immunization with a DNA vaccine (Wang *et al.* 1997). These data collectively suggest that an IgA antibody response can be achieved following local immunisation in the reproductive tract.

1.5.3 The effect of ovarian hormones on antibody levels in the reproductive tract fluid

Variations in Ig levels in the reproductive tract luminal fluids during the oestrous cycle have been observed in several species including rats, mice, horses and cattle. In these species, the concentrations of oestrogen and progesterone in blood change

cyclically with oestrogen being dominant before ovulation and progesterone being dominant after ovulation (Figure 1-5). The effect of ovarian hormones on IgA and IgG

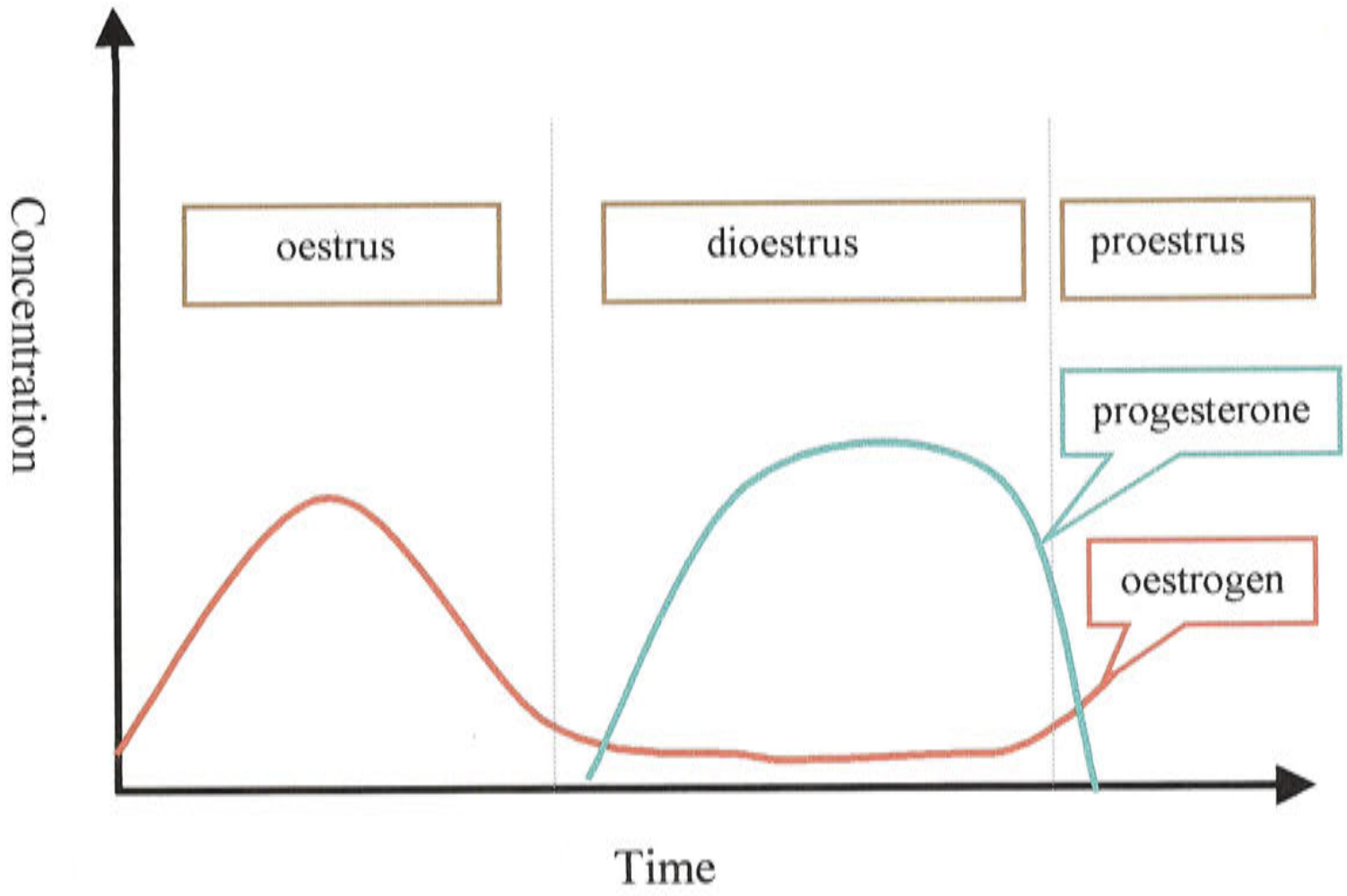


Figure 1- 5. The concentration change of oestrogen and progesterone during an oestrous cycle of mice, rats and livestock (adapted from Johnson and Everitt, 2000).

This diagram shows the concentrations of ovarian hormones in blood during an oestrous cycle of mice, rat and most livestock. At oestrus, oestrogen is the dominant hormone until ovulation. After ovulation, progesterone starts to increase in concentration and, during dioestrous, progesterone is the dominant hormone. At pro-oestrus, the progesterone level declines to a low level whereas the oestrogen level elevates in preparation for ovulation.

movement in the female reproductive tract has been well documented in the rat (Wira and Sandoe, 1978, 1980, 1987; Wira *et al.* 1984; Sullivan and Wira, 1984) where IgG and IgA levels in uterine sections increased during pro-estrus. IgA continued to increase at oestrus while IgG dropped. At dioestrus, both IgA and IgG were low (Wira and Sandoe, 1980). Oestradiol injection was followed by a transient increase in IgG levels shortly after the injection but a sustained increase in IgA levels in uterine tissues and secretions (Sullivan and Wira, 1984). This increase in IgA levels in the uterine fluids

has three possible sources: 1) an increase in the amount of IgA formed in the tissue and the infiltration of IgA-positive B cells into the endometrium or myometrium; 2) an increase in the rate of movement of IgA from the tissue into the uterine lumen; and 3) an increase in the amount of secretory component in the uterus (Wira and Sullivan, 1981). In the mouse, by contrast, IgA and IgG concentrations in uterine sections were highest at pro-oestrus, lower at oestrus, and were very low at dioestrus (Rachman *et al.* 1983). In addition, the levels of specific IgA antibody increased in female genital tract secretions after oestradiol treatment (Wang *et al.* 1996) or were significantly higher at oestrus than at other stages of the cycle (Gallichan and Rosenthal, 1996).

In contrast, specific IgG to HSV-1 in mouse vaginal washes was significantly higher after progesterone administration or at dioestrus (Gallichan and Rosenthal, 1996). In mares, the ratio of IgA to total protein in uterine secretions was significantly higher at oestrus than at dioestrus (Widders *et al*, 1985) and IgG concentrations in uterine fluids increased at dioestrous or after progesterone treatment (Mitchell *et al.* 1982, Johnson *et al.* 1994). A similar result was reported in cows (Whitmore and Archbald, 1977). There is, therefore, broad evidence that IgA levels in the reproductive tract are driven by oestrogen and are at their highest at those stages of the cycle when oestrogen levels are also high. In contrast, IgG levels appear to correlate with progesterone levels.

Unlike the species described above, the rabbit is an induced ovulator (Hammond and Marshall, 1925) with oestrogen being the dominant ovarian hormone until mating occurs. It is not clear how this will affect antibody levels in the reproductive tract fluids. Although a previous study showed that the total Ig level in the female rabbit reproductive tract fluids was slightly elevated after induction of ovulation (Oliphant *et al.* 1977), the effect of ovulation on specific antibody levels in the rabbit reproductive tract fluids has not been reported.

1.6 Immunocontraception targeting oocytes in the ovary

1.6.1 Infertility achieved by immunisation with zona pellucida protein

As mentioned in section 1.2.1, an advantage of targeting female rabbits for immunocontraception is that oocytes can provide alternative immunocontraceptive antigens. The zona pellucida (ZP) is a glycoprotein matrix that surrounds the oocyte and is the site of initial sperm binding to the ovulated oocyte. ZP proteins of oocytes are currently the most common oocyte antigens used for immunocontraception because of the special roles they play in oocyte development and fertilisation (Epifano and Dean, 1994). High infertility rates have been achieved in several species following immunisation with zona pellucida proteins (Sacco, 1987; Henderson *et al.* 1988; Skinner *et al.* 1996; Gupta *et al.* 1997). ZP proteins from both heterogenous and homogenous sources can be used for immunocontraception. Thus, porcine ZPC-α, the ZP protein that is considered to be the sperm receptor in the pig (Dunbar *et al.* 1994; Skinner *et al.* 1996), has been used as an immunocontraceptive antigen in monkeys in which high infertility rates were obtained (Sacco *et al.* 1987; Bagavant *et al.* 1994). Equally, immunisation of female mice with homogenous mouse ZP3 expressed in a recombinant virus also achieved a high infertility rate (Jackson *et al.* 1998; Chambers *et al.* 1999).

In female rabbits, immunisation with porcine ZP antigens resulted in a high rate of infertility (Jones *et al.* 1992), follicle loss (Skinner *et al.* 1984; Jones *et al.* 1992) and ovarian dysfunction (Skinner *et al.* 1984). But female rabbits immunised with native rabbit ZP protein showed no or a very low antibody response and there were very limited effects on fertility (Wood *et al.* 1981; Skinner *et al.* 1987). This suggested that immune tolerance existed to the self-antigen. Prasad *et al.* (1995) compared recombinant rabbit ZPB expressed in bacteria or in a baculovirus expression system and showed that eukaryotic-expressed, but not bacteria-expressed, ZP protein stimulated a strong antibody response in rabbits. A study conducted during my PhD course further demonstrated that subcutaneous immunisation with recombinant rabbit ZPB expressed in a rabbit cell line elicited a high-level antibody response in serum and resulted in an infertility rate of 75% in immunised rabbits (Kerr *et al.* 1999). Moreover, in this study it

was shown that rabbit ZPB delivered by a recombinant myxoma virus (MV-ZPB) was able to overcome self-tolerance to ZPB and induce an antibody response to this ZP antigen. However, the antibody response was transient and only 25% of rabbits were infertile (Kerr *et al.* 1999). On the other hand, immunisation of female mice with a recombinant ectromelia virus expressing mouse ZPC elicited a persistent antibody response to the antigen and resulted in a high infertility rate (Jackson *et al.* 1998). These results suggest that the glycosylation of the recombinant ZP protein is essential to enhance the immunogenicity of self-ZP antigen and that the recombinant virus delivery system provides a prospective way to overcome self-tolerance and achieve a high-level of infertility.

1.6.2 The mechanism of infertility following ZP immunisation

1.6.2.1 Antibodies blocking fertilisation

After immunisation with ZP, antibodies to ZP proteins could play an important role in blocking fertilisation by preventing sperm binding to oocytes. Antibodies to hamster ZP inhibited sperm attaching to and penetrating the zona pellucida (Shivers *et al.* 1972) and monoclonal antibodies against recombinant bonnet monkey ZPB inhibited the binding of human spermatozoa to human ZP in a hemizona assay (Govind *et al.* 2000), suggesting that antibody to certain epitopes of ZP can effectively prevent spermatozoa from interacting with ZP and so block fertilisation.

In theory then, antibodies to ZP in the oviduct can bind to ovulated oocytes and prevent fertilisation. However, in female rabbits immunised with sperm antigens, the antibody levels in the oviduct were not high (section 1.3.2). So how effective antibodies in the oviduct will be in blocking fertilisation is unclear. On the other hand, ZP antibodies in the ovary may be much more effective in binding to the oocyte and preventing sperm fertilising the eggs. Specific antibodies are able to bind to the surfaces of oocytes in ovarian follicles following immunisation with ZP antigens in mice (Tung *et al.* 1994; Jackson *et al.* 1998) and rabbits (Kerr, *et al.* 1999). Furthermore, passively injected ^{125}I labeled specific IgG to hamster ovarian antigens persisted in the ovary at a

high level and induced infertility but was not detectable in other tissues (Yanagimachi *et al.* 1976). These results collectively suggest that antibodies in the ovarian follicles could play a more important role than antibodies in oviductal fluids in blocking fertilisation. However, antibodies in the oviduct and the uterus could also play an important role in blocking embryo implantation.

1.6.2.2 Follicle loss, ovarian dysfunction and infertility

Immunisation with ZP antigens often induces follicle loss and ovarian dysfunction (Sacco, 1987; Henderson *et al.* 1988; Skinner *et al.* 1996). In the rabbit (Wood *et al.* 1981; Skinner *et al.* 1984; Jones *et al.* 1992; Kerr *et al.* 1999) and some other species, follicle disruption and ovarian dysfunction were closely related to infertility (mice, Jackson *et al.* 1998; marmoset monkey, Aitken *et al.* 1996; bonnet monkeys, Bamezai *et al.* 1986, and hamster, Hasegawa *et al.* 1992). However, oophoritis was not correlated to the infertility rate in mice and a specific T cell response to mouse ZPC was compatible with normal ovarian function (Bagavant *et al.* 1999). Thus, the relationship between follicle loss, ovarian dysfunction and infertility is still unclear.

The factors responsible for the ovarian pathology following ZP immunisation are still not fully understood. Studies in rabbits showed that antibody could play a principal role in follicle loss or ovarian pathology (Skinner *et al.* 1984; Kerr *et al.* 1999). Antibody binding to ZP of oocytes in follicles was suggested as the main cause of follicle disruption and ovarian dysfunction by interfering with the oocyte or follicle development (Skinner *et al.* 1984). However, how antibody could do this remains unclear. On the other hand, T cell responses could also play an important role in ovarian pathology. Immunisation of an inbred mouse strain with a mouse ZP3 peptide induced a specific CD4+ T cell response and these CD4+ cells alone could cause oophoritis, as demonstrated by a passive transfer experiment in which CD4+ but not CD8+ T cells were able to induce oophoritis in the recipients (Tung and Teuscher, 1995; Tung *et al.* 2001; Garza, *et al.*1998). This result suggested that under some circumstances T cell responses might play a role in ovarian pathology following ZP immunisation. However, the oophoritis was transient and, following recovery, could not be reinduced by boosting.

Immunocontraception that induces ovarian pathology is not suitable for human application and efforts in human immunocontraception are concentrated on finding B cell epitopes that could induce infertility without ovarian pathology (Govind *et al.* 2000). However, for control of a pest animal such as the European rabbit in Australia, infertility resulting from ovarian pathology following ZP immunisation may be an acceptable and long-lasting approach.

1.6.3 Antibody titers in ovarian follicles: the ovary could be more accessible to serum antibody

Clearly, if a ZP antigen is to be vectored for immunocontraception for rabbits, induction of a high level immune response in the ovary or oviduct is important. Whether recombinant myxoma virus can deliver a strong immune response in the ovary has not been determined. However, it seems probable that the ovary is more accessible to serum antibody than the mucosal compartment of the reproductive tract. Early studies in rabbits showed that the IgG concentration (Symons and Herbert, 1971) and the specific IgG titer to sperm antigen LDH-C4 (Kille and Goldberg, 1979) in serum was comparable to that in ovarian follicle fluids. In humans, anti-sperm IgG and IgA levels in follicular fluids were also similar to those in serum although IgM levels were lower (Clarke *et al.* 1984, Kay *et al.* 1985). Antibody levels in bovine ovarian follicle fluids were also shown to parallel those in serum (Whitemore and Archbald, 1977). In support of the idea that antibody titres in serum are important to ovarian pathology and infertility, studies in the rabbit showed that infertility rates were correlated with serum antibody titers (Skinner *et al.* 1984; Kerr *et al.* 1999). Serum antibody levels also correlated with ovarian dysfunction in squirrel monkeys (Sacco, 1987), follicle disruption and infertility in mice (Jackson *et al.* 1998) and infertility in bonnet monkeys (Bamezai, 1986). However, whether antibody titers in the follicles or reproductive tract are the underlying cause of infertility has not been determined.

1.7 The aim of this study

An effective immunocontraceptive vaccine for European rabbit population control needs both effective vectors and effective immunocontraceptive antigens. Myxoma virus has been chosen as a potential viral vector because of its species-specificity, well-known genetic background and transmission characteristics (Tyndale-Biscoe, 1994; Holland and Jackson. 1994; Kerr and Jackson, 1995). For the viral vector to be successful, it is vital that it can deliver a sufficiently strong immune response in the appropriate tissues (the mucosal compartment of the reproductive tract or the ovarian follicular fluid) so that fertilisation is blocked. An investigation of whether the recombinant myxoma virus could achieve this is a crucial step for developing viral vectored immunocontraception in rabbits. The selection of the immunocontraceptive antigen is also dependent on the nature of the immune response induced by the vector. If the virus can induce a strong antibody response in the mucosal part of the female reproductive tract then sperm antigens can potentially be used for immunocontraception. However, if the virus is not able to do this, then induction of a strong immune response in the ovarian follicles could be more important and this will require an oocyte or follicular antigen. In this study, the following investigations of the immune response in the female rabbits are described.

1). An examination of the distribution of immune cells in the female rabbit reproductive tract and response to ovulation.

2). A determination of the antibody response in the rabbit reproductive tract following immunisations by intradermal, intranasal and intra-vaginal routes with a recombinant myxoma virus expressing the model HA antigen.

3). An examination of the mechanism of the immune response in the rabbit ovary following immunisation with myxoma virus expressing the rabbit ZPB glycoprotein.

CHAPTER 2: MATERIALS AND METHODS

2.1 Biochemical reagents and solutions

2.1.1 Recombinant myxoma viruses and antigens

2.1.1.1 Recombinant myxoma viruses

Recombinant myxoma virus expressing influenza haemagglutinin (HA) (MV-HA) was described previously (Kerr and Jackson, 1995). The recombinant virus is derived from the Uriarra strain of myxoma virus and is extremely attenuated causing minimal disease in immunised rabbits. The HA cDNA is expressed under the control of a vaccinia virus late promoter, P11.

Recombinant myxoma virus encoding rabbit zona pellucida protein B (MV-ZPB) was described previously (Kerr *et al.* 1999). The RC55 cDNA (rabbit zona pellucida protein B gene) was cloned into the exact same site as HA. Similarly to the MV-HA recombinant virus the MV-ZPB virus is highly attenuated.

2.1.1.2 HA and MV antigens

A preparation of whole influenza virus (strain A/PR/8/34; supplied by Dr. Sonja Best, CSIRO Wildlife and Ecology) was used as antigen to measure specific IgG, IgM, and IgA antibody response to the HA antigen. This preparation was also used for intra-vaginal boosting and as the antigen for immuno-histochemical analysis of frozen tissue sections to quantify the number of cells producing anti-HA antibody.

A preparation of whole myxoma virus of Lausanne strain (gift from Louise Silver, CSIRO Wildlife and Ecology) concentrated from cell culture was used as antigen in ELISA to measure specific antibodies against myxoma virus.

2.1.1.3 Recombinant rabbit ZPB (RC55) protein

Rabbit ZPB protein was expressed in rabbit RK13 cells infected with vaccinia ZPB (Kerr *et al.* 1999). The ZPB protein was purified by affinity column chromatography using monoclonal antibody IG7E11 (supplied by Nigel French and Hannah Clarke, CSIRO Wildlife and Ecology).

2.1.2 Antibodies and antibody conjugates

The monoclonal antibodies, polyclonal antibodies and antibody conjugates used in this study are listed in the tables below (Table2-1, Table 2-2, and Table 2-3). Their specificities and manufacturers or references are also listed in the tables. Antibodies were aliquoted and stored according to the supplier's instruction.

Table 2- 1. Monoclonal antibodies used in this study

Monoclonal antibody	Specificity	Manufacturer or reference
Anti-MV (clone 3B6E4)	Myxoma virus	Fountain *et al.* 1997
Anti-rabbit Ig μ chain	B cells	SeroTec
Anti-rabbit CD4	T cell subset	SeroTec
Anti-rabbit KEN-5	T cells	SeroTec, (Kotani *et al.* 1993; Mage, 1998)
Anti-rabbit CD43	Leukosialin, mainly T cells, weak monocytes	SeroTec, (Jackson *et al.* 1983, Wilkinson *et al.* 1992)
Anti-rabbit CD45	Pan leucocyte	SeroTec, (Jackson *et al.* 1983)
Anti-rabbit MHC class II	MHC II R-DQ	SeroTec, (Lobel *et al.* 1984)

Table 2- 2. Polyclonal antibodies used in this study

Polyclonal antibodies	Specificity	Manufacturer
Goat anti-rabbit IgA	Ig α chain Fc fragment specific	Nordic
Goat anti-rabbit IgG	Ig γ chain specific	Nordic
Goat anti-rabbit IgM	Ig μ chain Fc fragment specific	Nordic
Rat anti-HA sera	Influenza virus	Supplied by Dr. Sonja Best
Sheep anti-mouse IgG sera	Mouse IgG	Boehringer Mannheim

Table 2- 3. Antibody conjugates used in this study

Conjugates	Specificity	Manufacturer
Goat anti-rabbit IgA conjugated with HRP[a]	Ig α chain Fc fragment specific	Nordic
Goat anti-rabbit IgG conjugated with HRP	Ig γ chain specific	Southern Biotechnology Associates, Inc.
Goat anti-rabbit IgM conjugated with HRP	Ig μ chain Fc fragment specific	Nordic
Goat anti-rat IgG conjugated with FITC[b]	Rat IgG (mouse adsorbed)	Silenus
Goat anti-rat IgG conjugated with Texas Red	Rat IgG (H+L)	Molecular Probes
Rabbit anti-goat Ig conjugated with FITC	Goat Ig	Nordic
Sheep anti-mouse IgG conjugated with FITC	Mouse IgG	Silenus
Sheep anti-mouse IgG conjugated with rhodamine	Mouse IgG	Silenus
Sheep anti-rat IgG conjugated with FITC	Rat IgG	Silenus

a: HRP: horse radish peroxidase; b: FITC: fluorescein isothiocyanate.

These antibodies or antibody conjugates were used to measure specific antibodies to HA, MV and ZPB or total immunoglobulins in serum and reproductive tract fluid samples. They were also used to label immunocyte surface markers in frozen sections of the reproductive tract tissues and ovary, and identify rabbit IgG, IgA and IgM in ovary.

2.1.3 Chemicals and solutions

Chemicals used in this study were purchased from Sigma or BDH Merck Pty, Australia and were all at or above analytic grade. For RNA work, freshly purchased chemicals or specialised RNA chemicals were purchased and stored for this purpose. All aqueous solutions were prepared in de-ionised or Milli-Q water. The commonly used solutions in this study and their components are listed below.

2.1.3.1 ELISA solutions:

PBS stock:	136 mM NaCl 2.68 mM KCl 8.09 mM Na_2HPO_4· 2.93 mM KH_2PO_4 pH=7.2, prepared as a 10x concentrate, autoclaved and stored at room temperature. 1x PBS was used as washing or coating buffer.
Carbonate coating buffer:	14.1 mM Na_2CO_3 34.9 mM $NaHCO_3$ Stored at 4°C
Colour reaction buffer:	18 mM ABTS (2, 2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, Sigma), 0.5 M citric acid, 0.6 M Na_2HPO_4, and 21 mM H_2O_2

2.1.3.2 Tissue or section fixation and slide preparation

Bouin's fixation solution:	0.9% picric acid (w/v) 9% formaldehyde (v/v) 5% acetic acid (v/v)
Formalin-calcium:	0.1 M $CaCl_2$ 1.33 M formaldehyde stored at 4°C
Poly-L-lysine solution:	100 mg poly-L-lysine (hydrobromide, Sigma) dissolved in 10 ml H_2O (w/v), stored in 2 ml aliquots at –20°C. Slide coating solution: 2 ml diluted into 20 ml H_2O.

2.1.3.3 Plasmid DNA preparation and sub-cloning solutions:

LB medium:	1% tryptone (w/v) 0.5% yeast extract (w/v) 0.17 M NaCl pH=7.2-7.4, autoclaved and stored at 4°C
5x TBE:	0.45 M Tris 0.44 M H_3BO_3 (boric acid) 0.1 M EDTA pH=8.0
Solution I:	50 mM D-glucose 25 mM Tris 10 mM EDTA pH=8.0 Autoclaved and stored at room temperature

Solution II:	0.2 M NaOH 1% SDS (Sodium dodecyl sulphate) Fresh made for use
Solution III:	3 M potassium acetate 11.5 % acetic acid Store at 4°C
Hydrolysis carbonate buffer:	60 mM Na_2CO_3 40 mM Na_2HCO_3 pH = 10.2, in diethyl pyrocarbonate (DEPC) treated H_2O
10x Hydrolysis neutralisation buffer:	2 M sodium acetate 10% acetic acid pH=6.0, in DEPC H_2O
20x SSC:	150 mM NaCl 15mM sodium citrate
TE buffer	10 mM Tris-HCl, pH=8.0 1 mM EDTA
T4 DNA ligase diluent:	20 mM Tris-HCl, pH=7.6 60 mM KCl 5 mM DTT (1,4-dithiothreitol) 0.05% BSA (Bovine serum albumin; w/v) 50% glycerol (v/v)

2.1.3.4 In situ hybridisation

50x Denhardt's:	1% ficoll (v/v) 1% polyvinylpyrrolidone (PVP, w/v)

	1% BSA (w/v) filtered and aliquoted 50 ml, stored at -20°C
Buffer 1:	0.1 M maleic acid 0.15 M NaCl pH=7.5 with NaOH autoclaved and stored at room temperature
Buffer 2:	10% blocking reagent (RNA Labelling and Detection Kit, Boehringer Mannheim) in Buffer 1, autoclaved and aliquoted for storage in a freezer.
Buffer 3:	100 mM Tris 100 mM NaCl 5 mM $MgCl_2$ pH=9.5

2.2 Animal treatment and sampling

2.2.1 Animal housing

Female New Zealand White rabbits aged 7-13 months old were used in this study. The animals were bred and housed in the animal house at CSIRO Wildlife and Ecology, Australia. Animal immunisations and other treatments were approved by the CSIRO Wildlife and Ecology animal ethics committee under NHMRC/CSIRO guidelines. Animal experiments related to recombinant myxoma virus were carried out in the PC2 facility within the animal house.

2.2.2 Immunisation and induction of ovulation

2.2.2.1 Immunisations

Intra-dermal immunisation with recombinant viruses and injections of rabbit ZPB protein for boosting were carried out by injection into the shaved skin of the rear leg. A total volume of 0.15 ml containing $1x10^6$ plaque-forming units (pfu) of myxoma recombinant virus MV-HA (Kerr and Jackson, 1995) or $1x10^3$ pfu of MV-ZPB (Kerr *et al.* 1999) was administered to each rabbit. For ZPB boosting, 100 mg purified recombinant ZPB protein in Freund's incomplete adjuvant (Sigma) in a total volume of 0.2 ml was similarly injected subcutaneously in two or three spots (Kerr *et al.* 1999).

Intra-nasal and intra-vaginal immunisations and intra-vaginal boosts were administered after the rabbits were anaesthetised by intra-muscular injection of Xylaze (5 mg xylazine/kg, Parnell Laboratory Pty Ltd, NSW Australia) followed by intravenous injection of Zoletil-100 (15 mg/kg, containing Tiletamine and Zolazepam at the concentration of 100 mg/ml, Virbac Pty, Ltd, Peakhurst, NSW Australia) 5 minutes later. At these doses animals remained anaesthetised for 1-2 hours. For intra-nasal immunisation, a total volume of 0.25 ml containing $1x10^6$ pfu MV-HA was given in small drops one by one into one nostril of the nose by pipette. For intra-vaginal immunization with MV-HA and intra-vaginal boost with influenza virus (HA), the rabbit was put on its side, a soft plastic transfer pipette (Sarstedt, Germany) containing the volume of virus was gently inserted into the vagina. When the tip reached the cervix, the dose was expelled. For immunisation, $1x10^6$ pfu MV-HA in a total volume of 0.25 ml was administrated. For intra-vaginal boost, 5000 influenza virus HA units /rabbit (diluted from a stock of $1.6x\ 10^8$ HA units/ml) in incomplete Freund's adjuvant was administered.

2.2.2.2 Induction of ovulation and super-ovulation

Ovulation was induced in rabbits by intravenous injection of 150 international units (i.u) human Chorionic Gonadotrophin (hCG, Chorulon ® Chorionic Gonadotrophin, Intervet Pty, Ltd NSW, Australia) 12 hours before sampling (Otsuki *et al.* 1990). Super-ovulation was induced by intra-muscular injection of 150 i.u/rabbit of PMSG

(PREGNECOL Injection Serum Gonadotrophin, Heriot, Agvet Pty, Ltd, Rowville Vic, Australia) 72 hours before sampling and intravenous injection of 125 i.u/rabbit hCG 12 hours before sampling.

2.2.3 Sample collection and preparation

2.2.3.1 Serum, reproductive tract fluids and ovarian follicular fluids

Blood samples were taken from a marginal ear-vein and allowed to clot at room temperature for 0.5-1 hour for preparation of serum. Serum was separated by centrifuging in a microcentrifuge at 15000 rpm for 5 minutes and was stored at –20°C for analysis.

Reproductive tract fluids were collected by two methods, micropuncture and flushing. For collecting free fluids, the rabbits were anaesthetised with pentobarbitone (Parnell Laboratory Pty Ltd, NSW Australia) and samples of the fluids in the lumen of oviduct, uterus and vagina were collected by Dr. Russell Jones (The University of Newcastle, Australia) using micropuncture with capillary tubes as previously described (Sujarit *et al.* 1990). For collection of flushing samples, rabbits were killed and the whole female reproductive tract was isolated. The oviduct and uterus from one side of the reproductive tract as well as most of the vagina were each flushed with 1 ml sterilised PBS and the flushing fluids were collected and stored at –20°C until analysis.

Vaginal and nasal washings were collected by flushing the vagina with 1 ml sterilised PBS and flushing the nasal cavity with 0.5 ml PBS after the rabbits were anaesthetised.

Ovarian follicular fluids were collected after induction of super-ovulation (section 2.2.2.2). The rabbits were killed and the ovaries were isolated. The follicular fluids in some pre-ovulatory follicles could be seen clearly with the naked eye and were collected directly by puncturing the follicles with capillary tubes. For some smaller follicles, the fluids were collected under a stereo microscope (SZ-PT, Olympus, Japan)

with a capillary tube and all collections from each rabbit were pooled. For each ovary, 20-50 µl fluid could be collected. Heparin (5 µl of 2000 u/ml solution) was added to each fluid sample to prevent clotting.

2.2.3.2 Reproductive tract tissue and other tissue samples

To examine cellular responses and histological changes in the reproductive tract, tissue samples were collected for preparation of frozen and paraffin-embedded sections. Oviductal and uterine tissues were collected from the non-flushed side of the tract. Oviduct at the middle of ampulla and uterus at the middle of uterine body were collected. Whole cervix and a part of vagina 1 cm from the cervical conjunction were collected. Whole ovaries were collected; left side ovaries for frozen sections and right side ovaries for paraffin embedded sections. A part of the popliteal lymph node from the injected side of intra-dermally immunised rabbits and uninfected control rabbits was also collected for preparation of frozen sections as positive controls.

2.2.3.3 Preparation of frozen sections

Immediately after collection, tissue samples were embedded in Tissue-Tek, O.C.T. Compound (Miles Inc. Elkhart, USA) in specimen molds (Cryomold 15mm+15mm +5mm, Miles Inc. Elkhart, USA) for fixation and freezing at -70°C. When sectioning, the tissue was trimmed on a cryostat (Lipshaw Company, USA) until good cross sections could be cut. Slides used for frozen sections were pre-coated with poly-L-lysine solution (section 2.1.3.2). Serial sections were then collected onto slides, which were labeled serially. Immediately after sectioning the sections were fixed in formalin-calcium (section 2.1.3.2) and acetone-chloroform mixture (1:1 volume) for 5 minutes each. The sections were washed 3 times in PBS, air dried in a fume hood, and stored at –70°C until staining.

2.2.3.4 Paraffin embedded section

Tissue samples were directly transferred into 4 ml Bouin's fixation solution (section 2.1.3.2) for about 4 hours. The Bouin's fixation solution was then replaced with 4 ml 70% alcohol, which was changed several times. Paraffin embedded sections were

prepared by the Department of Histology of John Curtin School of Medical Research (The Australian National University, Canberra, Australia) and stained with haematoxylin and eosin (H&E).

2.3 Antibody and immunoglobulin measurement

2.3.1 Antibody measurement

ELISA was used to measure specific IgG, IgM and IgA to HA and myxoma virus. Antigen, either influenza virus or myxoma virus, was diluted in PBS (1:5000 for HA antigen; 1:2000 for myxoma virus antigen) and 50 µl antigen solution was transferred to each well of 96-well micro-plates (Greiner, Labortechnik) for coating at 37°C for 3 hours. The plates were washed 3 times at room temperature and blocked at 4°C overnight with 3% skim milk. Serum or fluid samples thawed at 4°C were centrifuged in a microcentrifuge at 13500 rpm for 5 minutes and then serially diluted in PBS in a 2-fold series. For serum, dilutions started at 1:50 and for reproductive tract fluid samples dilutions started at 1:5. For specific IgA measurement, the initial dilution was 1:3. The diluted sample (50 µl) was added to duplicate wells. The plates were incubated at 37°C for 2 hours for antibody binding followed by washing 4 times with 0.1% (v/v) Tween-20 in PBS (PBST). Antibody binding was detected by adding 50 µl of the second antibody conjugate, sheep anti-rabbit IgG conjugated with HRP (1:5000 dilution) or goat anti-rabbit IgA or IgM conjugated with HRP (1:1000 dilution). After 1-hour incubation and 6 washes with PBST, 100 µl ABTS solution (section 2.1.3.1) was added to each well for the colour reaction. The plates were read at 405 nm on a Bio-Rad Micro-plate Reader (Bio-Rad, Model 3550). The antibody titre was determined by the highest dilution that gave an absorbence value more than 0.1 above the normal serum control (absorbence values for blank wells and negative controls were usually 0.005-0.02). Positive serum from a rabbit infected with recombinant virus MV-HA and negative serum from a normal uninfected rabbit (supplied by Dr. Sonja Best) were used as positive and negative controls (1:100 dilution). Blank controls were wells without antigen.

ELISA was also used for measuring antibody titers to rabbit ZPB as previously described (Kerr *et al.* 1999). Briefly, plates were coated with purified recombinant rabbit ZPB protein (2 μg/well) with carbonate coating buffer (section 2.1.3.1). The plates were then washed and blocked with 3% skim milk/PBS. Serum samples or ovarian follicular fluids were serially diluted and added to the wells as described above. The plate washings, antibody conjugate application, colour reaction and plate reading were precisely as described above.

2.3.2 Total immunoglobulin measurement

2.3.2.1 IgG and IgM

Direct ELISA was used to measure total IgG and IgM in serum and reproductive tract fluids. The samples were diluted in PBS in a 2-fold series as described in section 2.3.1 and 50 μl of each dilution was transferred to a micro-plate for absorption at 37°C for 2 hours and then at 4°C overnight. The plates were washed and blocked as in section 2.3.1. Goat anti-rabbit IgG or IgM conjugated with HRP was diluted in 1% skim milk (anti-IgG 1:4000, anti-IgM 1:1000) and 50 μl was added to each well for incubation at 37°C for 1 hour. The colour reaction and plate reading were as in section 2.3.1. Negative control was 3% BSA.

2.3.2.2 Total IgA

Immuno-diffusion was used to measure total IgA in serum and reproductive fluids. Fifteen ml of 1% agarose was poured into a 15 cm Petri dish. Rosette holes (six holes surrounding one central hole) were punched after the agarose was set. The central hole was filled with 20 μl goat-anti-rabbit IgA antibody and surrounding holes were filled with 20 μl samples that were diluted in PBS in a 2 fold series starting at 1:2. The dish was incubated at 37°C for 24 hours after which precipitate bands were measured. Positive controls were rabbit serum or rabbit IgA or IgG standards (Sigma). Negative control was 3% BSA. The titer was determined by the highest dilution giving a clear precipitate band.

2.4 Immunofluorescence

2.4.1 Indirect immunofluorescence

Indirect immunofluorescence was used as a general method to label rabbit CD45, CD43, KEN-5, CD4, immunoglobulin μ-chain, MHC class II positive cells and IgG and IgA-containing cells in frozen sections of reproductive tract tissues, small intestine tissue and lymph nodes. This method was also used to localise rabbit IgG, IgM and IgA in frozen sections of the ovary.

The frozen sections prepared and stored at –70°C (section 2.2.3.3) were air dried at room temperature for 1 hour. The sections were then blocked with 200 μl 3% BSA/PBS for 1 hour at room temperature in a humidity box. After 3 washes with PBS, 50 μl of primary antibodies diluted in 1% BSA/PBS (1:10 dilution for monoclonal antibodies and 1:50 dilution for polyclonal antibodies) were applied to each section for an incubation of two hours at 37°C in a humidity box. After a further 4 washes with PBS, the second antibodies, sheep anti-mouse IgG (for monoclonal antibody as primary antibody) or rabbit anti-goat Ig (for polyclonal antibody as primary antibody) conjugated with FITC (Table 2-3) were diluted 1:50 in 1% BSA/PBS and 50 μl was applied to each section. The negative control was 1:100 mouse ascites fluid (MAF) or 1:100 normal goat sera (Silenus). Positive controls were the frozen sections from popliteal lymph nodes or small intestine (for IgG and IgA containing cells). The sections were incubated at 37°C for one hour in a humidity box covered with aluminium foil to exclude light; washed 4 times with PBS and mounted with anti-fade solution (Molecular Probe Inc. USA). The sections were kept at 4°C covered with foil until confocal microscopy (Bio-Rad, MRC 1000).

2.4.2 Immunofluorescence for detection of HA specific antibody secreting cells

Frozen tissue sections were air dried and blocked as in 2.4.1. After washing in PBS, the sections were incubated with influenza virus (1:2000 dilution of a stock of 1.6×10^8

HA unit/ml) for 1 hour at 37°C. The sections were washed 4 times in PBS and incubated with rat anti-HA antibody (kindly supplied by Dr. Sonja Best) at 37°C for 2 hours. After an additional 4 washes in PBS the sections were incubated with rabbit anti-rat IgG conjugated with FITC (Table 2-3) at 37°C for 1 hour in a humidity box in the dark. The sections were then washed in PBS and mounted with anti-fade solution as described above. Each slide was viewed by confocal microscopy. Frozen sections of popliteal lymph nodes draining the inoculation side of intradermally immunised rabbits were used as positive controls. Frozen sections of reproductive tract from uninfected controls were used as negative controls. Other controls included omitting influenza virus antigen or replacing primary antibody with normal rat serum.

2.4.3 Double immunofluorescence

Double immunofluorescence was used to label two cell surface markers on frozen sections. This method was used to double-label rabbit KEN-5 and MHC class II and MHC class II and myxoma virus positive cells. Two antibody conjugates, one with FITC (green) and another with rhodamine (red) were used.

The sections were air dried and blocked as in 2.4.1. After 3 washes in PBS, 50 µl of the first monoclonal antibody diluted 1:10 in 1% BSA/PBS (e.g to rabbit KEN-5 or MHC class II) was applied to each section. The sections were incubated at 37°C for 1 hour in a humidity box. After 4 washes in PBS, 50µl of the first fluorescent conjugate, sheep anti-mouse IgG conjugated with FITC (green, Table 2-3), diluted in 1%BSA/PBS (1:25), was applied to each section. The sections were incubated at 37°C for 45 minutes in a humidity box in the dark. After another 4 washes in PBS (kept in the dark as much as possible), any unbound mouse IgG was blocked by incubation with 100 µl 1:50 diluted (in 1% BSA/PBS) sheep anti-mouse IgG serum (Table 2-2) at 37°C for 1 hour; followed by a further 4 washes in PBS (kept in dark). The second monoclonal antibody (eg. to rabbit MHC class II or myxoma virus) was diluted 1:10 in 1% BSA/PBS and 50 µl was applied to each section. The sections were then incubated at 37°C for 1 hour in a humidity box in the dark. After a further 4 washes in PBS (kept in the dark), 50µl of the second fluorescent conjugate, sheep anti-mouse IgG conjugated with rhodamine (red, Table 2-3) diluted 1:10 in 1% BSA/PBS, was applied to each section. After incubation

at 37°C for 45 minutes in a humidity box in the dark, the sections were then washed in PBS 4 times, followed by anti-fade treatment as in 2.4.1.

2.4.4 Cell counting using confocal microscopy

The immunofluorescently stained and mounted slides were viewed by confocal microscopy. Images were captured electronically. Constant parameters were used while counting the cells, e.g. zoom 2.0, iris 4.0, gain 1200, laser beam 30%, back-level 5, and enhancer 0. Three representative areas in each section were counted. The histogram function of the management software (Bio-Rad) was used to outline the areas to be counted and calculate the area.

The areas for cell counting of each region of the reproductive tract was defined as: oviduct: whole villi, including epithelium and laminal propria; uterus: epithelium and the first half of the endometrium; cervix: similar to uterus; vagina: whole mucosa area, including epithelium and lamina propria.

2.5 Preparation of sense and anti-sense RNA probes

RNA probes of rabbit IL-2 were prepared for detection of mRNA expression in tissue samples. Sense and anti-sense RNA probes were prepared. Anti-sense probes would hybridise with the RNA of interest. Sense probes were used as control probes that would not hybridise with the RNA of interest.

2.5.1 Sub-cloning IL-2 DNA

The purpose of sub-cloning was to insert the IL-2 cDNA fragment into two plasmids under the control of the same promoter but in opposite orientations. This allows transcription from the same promoter and hence synthesis of equal amounts of sense

and anti-sense RNA probes. Full-length rabbit IL-2 cDNA was sub-cloned into pGEM-T (Promega) under the control of the T7 promoter or into Bluescript SK+/- (Promega) also under the control of the T7 promoter but in the opposite orientation.

2.5.1.1 Mini-preparations of plasmid DNA

Mini-preparations of plasmid DNA were made as described by Sambrook *et al.* (1989). Briefly, a single colony of TG1 *E. coli* that contained pGEM-T-IL-2 or BluescriptSK+/- was inoculated into 2 ml LB medium (section 2.1.3.4) and cultured at 37°C overnight on a shaker. The cells were transferred into a 1.5 ml tube and pelleted in a micro-centrifuge at 13500 rpm for 30 seconds. The cell pellet was resuspended in 100 µl ice-cold Solution I (section 2.1.3.4) by vortexing and the cells were lysed by adding 200 µl freshly made Solution II (section 2.1.3.4) with gentle mixing. After 5 minutes incubation on ice, 150 µl of ice-cold solution III (section 2.1.3.4) was added and the tube inverted twice to mix followed by incubation in an ice-bath for 5 minutes and centrifuging at 13500 rpm in a micro-centrifuge for 5 minutes. The supernatant was transferred into a new tube and an equal volume of phenol: chloroform (1:1, v/v) was added and vortexed. The aqueous phase was separated by centrifuging at 13500 rpm for 2 minutes and transferred to another tube. Two volumes of 100% ethanol were added and mixed and the tube was kept at room temperature for 2 minutes and then on ice for 5 minutes. Plasmid DNA was precipitated by centrifuging at 13500 rpm for 15 minutes. The DNA pellet was washed with 1 ml 70% ethanol at room temperature and air dried for about 15 minutes and then was resuspended in 50 µl TE buffer (pH=8.0) containing 20 µg/ml RNase A (DNase free RNase, Promega). The concentration of DNA was determined by reading the OD value at 260 nm on a spectrophotometer (Varian).

2.5.1.2 Restriction enzyme digestion for ligation

Restriction digestions were conducted to linearize the BluescriptSK+/- plasmid and cut IL-2 DNA fragment from the pGEM-T for ligation. Two restriction enzymes, Sac II and Sal I were used for both digestion reactions and the reactions were set up in a 30 µl volume in 1.5 ml tubes.

Plasmid DNA:	4 µl
H_2O	22 µl
10x Buffer	3 µl
Sac II	1 µl (2 u)

The reactions were incubated in a 37°C water bath for three hours. A volume of 5 µl was taken for agarose gel electrophoresis. The reactions were continued by adding 2.5 µl 10x Buffer and 1 µl Sal I (2 u) to the tubes followed by incubation at 37°C for another 3 hours. A mini agarose gel (1% in TBE Buffer) was used to check linearization of the plasmid and also for isolating the IL-2 DNA fragment from the agarose.

2.5.1.3 Purification of DNA fragment

After agarose gel electrophoresis, the DNA bands of the correct size for the rabbit IL-2 DNA and the linearised BluescriptSK+/- plasmid were excised from the gel under UV light. The gel slices were weighed and Nucleotrap (Promega) was used to purify the DNA fragments from agarose following the protocol provided by the manufacturer.

2.5.1.4 Ligation reaction and transformation

A ligation reaction was set up to ligate the IL-2 cDNA fragment and linearized BluescriptSK+/- plasmid which had been digested with the same restriction enzymes. The reaction was set up as follows in a total volume of 20 µl.

Plasmid DNA	3 µl
Rabbit IL-2 cDNA fragment	3 µl
H_2O	11 µl
Ligation buffer (10x)	2 µl
T4 DNA ligase	1 µl (diluted as 2 u/µl)

The reaction was incubated in a 16°C water bath for 4 hours.

After the ligation reaction, the products were transformed into *E. coli strain* TG1 competent cells (supplied by Shanshan Wu, Wildlife and Ecology, CSIRO). A volume

of 0.9 ml ice-cold 0.1 M $CaCl_2$ was added to 0.1 ml competent cells. After 2 minutes on ice, 100 µl of the competent cells were transferred into another pre-chilled 1.5 ml tube and 5 µl ligation reaction mixture was added. The tube was kept on ice for 25 minutes. During this period, the tube was swirled several times. The cells were then heat-shocked by incubation at 43.5°C in a water bath for 45 seconds and then kept on ice for 2 minutes. The cells were pelleted in a microcentrifuge for 30 seconds and resuspended in 100 µl LB medium. The cells were spread on a LB-agar (1.5% agar in LB medium) plate supplemented with ampicillin (50 µg/ml). Positive colonies were chosen, based on ampicillin resistance conferred by the Bluescript plasmid, and cultured for mini-preparation. The correct insertions of IL-2 DNA fragments were confirmed by restriction enzyme digestion and agarose gel electrophoresis.

2.5.2 Synthesising and labelling RNA probes

2.5.2.1 Preparation of sense and anti-sense DNA templates

To synthesise and label specific sense and anti-sense RNA probes, a purified DNA template is necessary. Plasmid DNA Preparation QIA gene Kit (Promega) was used to prepare sense and anti-sense DNA templates. Single colonies that contained sense and anti-sense plasmids of IL-2 were inoculated into 30 ml LB medium supplemented with 50 µg/ml ampicillin. The cells were cultured at 37°C overnight in a shaker and then pelleted by centrifuging at 5000 rpm (rotor AJ-20, Beckman) for 30 minutes. The manufacturer's protocol was followed to purify plasmid DNA. At the final step the DNA pellet was air dried and plasmid DNA was resuspended in 0.5 ml TE (pH=8.0) and aliquoted in small volumes for further use.

For preparation of sense and anti-sense IL-2 DNA templates, the IL-2/BluescriptSK+/- plasmid was digested with Sac II (sense templates) and the IL-2/pGEM-T plasmid was digested with Sal I (anti-sense templates) that allowed transcription to begin with the T7 promoter and stop at the end of the IL-2 DNA fragment. The digestion reactions were set up as in section 2.5.1.2 with 1 µg of plasmid DNA and 1 µl of the restriction enzyme (2-4 units) in a total volume of 20 µl in 1.5 ml tubes. The tubes were incubated at 37°C for 2 hours. After digestion, a sample (2 µl)

was taken from the reaction to check that digestion was complete by agarose gel electrophoresis. To recover the DNA template, 20 µl phenol: chloroform mixture (1:1 v/v) was added to the digestion reaction and vortexed thoroughly. The two phases were separated in a microcentrifuge at 13500 rpm for 5 minutes. The aqueous phase was transferred into a new tube and extracted with an equal volume of chloroform. The aqueous phase was again transferred to a new tube and the lid of the tube was left open for 5 minutes at room temperature in a fume hood to allow any remained chloroform to evaporate. DNA templates were recovered by adding 2 µl 3 M sodium acetate and 50 µl 100% ethanol to each tube and centrifuging in a microcentrifuge for 15 minutes. The pellet was washed with 50 µl 70% ethanol and air dried at room temperature for 10 minutes. The DNA template was resuspended in 13 µl RNase free water (DEPC water) for transcription.

2.5.2.2 Transcription and labelling

A non-radioactive DIG (Digoxigenin) RNA Labelling and Detection Kit (Boehringer Mannheim Biochemica) was used to synthesise and label the RNA probes. The transcription reactions were set up as following in a total volume of 20 µl.

Templates DNA	13 µl
10x Transcription Buffer	2 µl
NTP labelling mixture	2 µl
RNase Inhibitor	1 µl
RNA Polymerase	2 µl

The reactions were incubated in a 37°C water bath overnight. After labelling, 2 µl RNase free DNase (Promega) was added to each reaction to digest the DNA template at 37°C for 3 hours. A small volume was taken to check RNA synthesis and DNA template digestion by gel electrophoresis. For precipitating the RNA probe, 5 µl 4 M LiCl (made in DEPC water) and 75 µl 100% ethanol were added to each tube and the tubes were kept in a -20°C freezer for 2 hours followed by centrifugation in a microcentrifuge at 13500 rpm for 15 minutes. The probes were washed with 20 µl cold 80% ethanol and dried in air for 10 minutes.

2.5.3 RNA probe hydrolysis:

In order to reduce the size of the probes and thus allow better penetration into the cells, the RNA probes were chemically degraded by incubation in alkali at 60°C to reduce their size to about 50 bp. Following precipitation and air drying as above, the RNA pellet was resuspended in 100 µl RNase-free water. The hydrolysis reaction was conducted by adding 95 µl hydrolysis carbonate buffer (section 2.1.3.4) to each tube and incubating at 60°C for 30 minutes after which the reaction was stopped by adding 10 µl of 10x hydrolysis neutralisation buffer (section 2.1.3.4). To precipitate the RNA probes, 20 µl 10% acetic acid, 24 µl 3M sodium acetate, and 640 µl 100% ethanol were added to each tube and the tubes were placed in a -20°C freezer overnight. The RNA probes were precipitated by centrifuging at 13500 rpm for 15 minutes. The pellets were washed with 100 µl cold 80% ethanol, dried in air for 5 minutes, resuspended in 50 µl TE (pH8.0, RNase free) and aliquoted and stored at –20°C in pre-hybridisation solution (section 2.6) containing RNase inhibitor (2 µl/ml, Promega).

2.5.4 Concentration test

To test the result of labelling and determine the concentration of RNA probe, a spot analysis was used. Each RNA probe (sense and anti-sense) was serially diluted with a dilution solution (mixture of DEPC H_2O: 20x SSC: formaldehyde at a ratio of 5:3:2 v/v/v). A sample of 2 µl from each dilution was transferred onto a nylon membrane (Hybond-N^+, Amersham) including unlabelled controls and antibody control. The membrane was wetted in Buffer 1 (section 2.1.3.4) and then put in 10 ml Buffer 2 (section 2.1.3.4) for blocking at room temperature for 30 minutes on a shaker. The membrane was then incubated in 10 ml anti-DIG antibody solution (From the Kit, 1:5000 dilution in Buffer 1) at room temperature for 30 minutes with shaking. After 3 washes in Buffer 1 the membrane was transferred into a colour substrate solution which contained 10 ml buffer 3 (section 2.1.3.4), 45 µl NBT (nitroblue tetrazolium, vial No.9 of the Kit) and 35 µl X-phosphate (vial No. 10 of the Kit), for colour reaction (about 20 minutes) in dark. The concentration of each probe used for in situ hybridisation was determined by the highest dilution that gave a dark spot. This was usually between a 1 in 100 and a 1 in 200 dilution of the original probe.

2.6 In situ hybridisation

In situ hybridisation was used to detect IL-2 mRNA expression in frozen sections of reproductive tract tissue and lymph nodes. The protocol was basically adopted from a booklet "Nonradioactive in Situ Hybridisation Application Manual" (Boehringer Mannheim) with some modifications. This protocol was carried out with care to avoid RNase contamination until the hybridisation step.

The tissue sections were prepared as in section 2.2.3.3. After air drying, the sections were dehydrated in 70%, 90% and 100% ethanol, 2 minutes each, in a staining container and allowed to air-dry. Pre-hybridisation was conducted on each section before hybridisation, 100 µl pre-hybridisation solution was applied to each section and the sections were kept in a humidity box at 42°C for 5 hours. After this, the anti-sense probe was diluted 1:100 in pre-hybridisation solution and 100 µl was applied to each section. A sense probe was similarly diluted and applied to control sections. The sections were all covered with coverslips and incubated at 48°C overnight in a humidity box. After hybridisation, 5 washes at 42°C were applied and these consisted of 2 washes with 2x SSC, 1 wash with 0.2x SSC and 2 washes with 0.1x SSC. Each wash took 15 minutes.

Pre-hybridisation solution:

Stock solution	Required concentration	Make up 1 ml
Formamide	50%	500 µl
20×SSC	5×	250 µl
50xDenhardt's solution	5x	100µl
10 mg/ml tRNA	250 µg/ml	25 µl
10 mg/ml salmon sperm DNA	250 µg/ml	25 µl
1 u/µl RNAsin	2 u/ml	2 µl
H_2O		98 µl
Total volume		1000 µl

The immunological detection of hybridisation basically followed the instructions of the manufacturer (Dig Kit, Boehringer Mannheim). Briefly, 100 µl Buffer 1 (section 2.1.3.4) was applied to each section and the sections were kept at room temperature for 2 minutes. Then the sections were blocked by applying 100 µl Buffer 2 (section 2.1.3.4) to each section and incubating at 37°C for 30 minutes in a humidity box. The sections were then incubated with 100 µl antibody solution (1:5000 dilution in Buffer 2) at 37°C for another 30 minutes in a humidity box. After two washes in Buffer 1, 100 µl Buffer 3 (section 2.1.3.4) was added to each section and the sections were kept at room temperature for 5 minutes. The sections were then moved into 20 ml colour solution (section 2.5.4) for colour development in the dark. For the first 10 minutes the sections were kept in this solution without shaking; the sections were then checked under the microscope until the signal on the positive section (sections from lymph nodes) could be seen clearly (about 30-60 minutes). The colour reaction was stopped by putting the sections into TE buffer (section 2.1.3) for 10 minutes.

2.7 Data analysis and statistics

Antibody titers determined by ELISA were transformed into logarithms and expressed as Log_{10} titers in figures and tables. Student t-test (Microsoft, Excel) was used to test for differences between treated groups and untreated controls. A $P<0.05$ in a two tailed test was considered as significant.

CHAPTER 3: IMMUNE CELLS IN THE FEMALE RABBIT REPRODUCTIVE TRACT

3.1 Introduction

Most vaccine and immunological studies of the reproductive tract have been done using rodent models (Parr and Parr, 1997, 1998; Medaglini, *et al.* 1997; Wira and Sandoe, 1987; 1995, Xiang *et al.* 1999). These species have the advantage of having well-characterised and well-understood immune responses and agents for studying their immune responses are readily available.

The rabbit is much less well understood and very few reagents are available for its immunological characterisation. If we are to examine fertility control by immunological means in the reproductive tract of the female rabbit, it will first be necessary to characterize those reagents that are available for studying immune responses in rabbits and to contrast the rabbit reproductive tract with that of other species. Furthermore, since the rabbit is an induced ovulator rather than going through a cyclical oestrus, it is necessary to induce ovulation in order to study possible variations in immunological response in different reproductive states. In this chapter, a panel of monoclonal and polyclonal antibodies was used to investigate the presence and localisation of immune cells such as T cells, MHC class II positive cells, and B cells in the oviduct, uterus, cervix and vagina of the rabbit both before and after ovulation.

The identification and characterisation of rabbit immune cells is hampered by the fact that far fewer monoclonal antibodies to rabbit CD antigens are available than to those of mouse and human. A previous study on rabbits reported that a dominant

population of T cells, together with a few IgA plasma cells and macrophages, were present in the endometrium and that the T cell number increased following the induction of ovulation (Otsuki *et al.* 1990). Another study in rabbits showed that strong IgG staining was present in the lamina propria of the reproductive tract and IgG plasma cells were present in the vagina (Symons and Herbert, 1971). However, systematic and detailed information about immune cells such as T and B cells, as well as APC in the whole reproductive tract, is not available. This is important for understanding antigen presentation and immune response in the reproductive tract following immunisation and for developing strategies to stimulate an immune response in the reproductive tract.

T cells are the dominant lymphocytes in the female reproductive tract of humans (Morris *et al.* 1985; Fernandez-Shaw *et al.* 1995; Givan *et al.* 1997; Johansson *et al.* 1999), mice (Parr and Parr, 1991; Nandi and Allison, 1993), cows (Cobb and Watson, 1995) and mares (Watson and Thomson, 1996). A large proportion of the T cells present in the reproductive tract of these species was located within the epithelium (intra-epithelial lymphocytes, IEL). Both CD4+ (T helper) and CD8+ (T cytotoxic /suppressor) T cells were reported in the endometrium with CD8+ being the dominant population in human (Pace *et al.* 1991) and mice (Croy *et al.* 1993). However, in the vagina, CD4+ cells were more common than CD8+ cells in rhesus macaques (Miller *et al.* 1992) and were the dominant sub-population in the mouse (Fidel *et al.* 1996). These data suggest that the distribution of T cells in the female reproductive tract varies from region to region and from species to species. In rabbits, little information is available about T cells or T cell subsets in the different compartments of the female reproductive tract.

In contrast to T cells, only a few B cells are present in the female reproductive tract of most reported species (Parr and Parr, 1996). Nevertheless, IgG and IgA plasma cells were reported in the reproductive tract of female mice (Rachman *et al.* 1983; Parr and Parr, 1985) and IgA plasma cells together with a few IgG plasma cells were described in the human fallopian tube (Kutteh *et al.* 1990). Plasma cells are usually considered to contribute to local antibody production; they have not yet been studied in the reproductive tract of female rabbits.

Local APC in the reproductive tract probably play a crucial role in presenting antigenic peptides to T cells in the draining lymph nodes, thus initiating an immune response that allows effector cells to return to the reproductive tract (McGhee *et al.* 1994). These APC are MHC class II positive. Therefore, detecting MHC class II expression in the reproductive tract could provide information about the presence and localisation of APC. However, it must be borne in mind that other cells, including B cells, also express MHC class II and it has been suggested that rabbit T cells and neutrophils may also express MHC class II (Mage, 1998). In studies of rat (Kaushic *et al.* 1998) and human (Morris *et al.* 1985; Ljunggren and Anderson 1998; Johansson *et al.* 1999), MHC class II positive cells were reported in the female reproductive tract. In rabbits, although macrophages were found in the endometrium (Otsuki *et al.* 1990) their distribution in the lower reproductive tract and that of other APC in the whole reproductive tract remains unknown. To determine whether APCs are present in mucosal compartments of the rabbit reproductive tract and whether their distribution is affected by ovulation, the distribution of MHC class II positive cells in the female rabbit reproductive tract was examined.

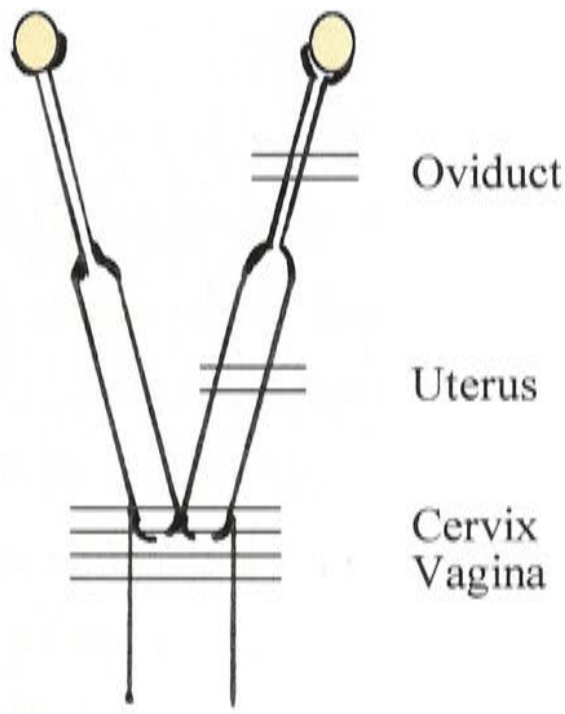
Studies in rodents and humans indicated that the stage of the oestrous cycle and the level of ovarian hormones had a significant effect on the number, location, and function of immune cells in the female reproductive tract (mice, Parr and Parr, 1991, rats, Wira and Rossoll, 1995; Kaushic *et al.* 1998; humans, Given *et al.* 1997; White *et al.* 1997). However, the rabbit is an induced ovulator and so does not have an oestrous cycle. This means that oestrogen is the dominant ovarian hormone in the rabbit until mating occurs; mating triggers follicle maturation, ovulation and the formation of the corpora lutea after which progesterone predominates. Although Otsuki (1990) showed that T cell number increased in the rabbit uterus after induction of ovulation, the effect of ovulation on T cells in other regions of the reproductive tract and on the distribution of other immune cells such as APC in the reproductive tract is still to be investigated.

There is considerable variation in the endocrine control of reproduction and in immune responses in the female reproductive tract. Furthermore, very little is known about either of these factors in the rabbit. Accordingly, this chapter describes an examination of potential immune response elements in the female rabbit reproductive tract, both before and after ovulation, to determine how these might differ, in both type

and location, from other species. This will provide us with the background necessary for later studies aimed at generating an immune response in the reproductive tract against a virally-delivered stimulator as a potential model of virally-vectored vaccination for fertility control.

3.2 Experimental design

The immunological status of the reproductive tract was assessed in two groups of rabbits. Four female rabbits aged 10-12 months which had been kept apart from males ('normal rabbits') formed the control group. To determine the effect of ovulation on immune cell distribution, four female rabbits of similar age (ovulated group) were injected with hCG (Chapter 2 section 2.2.2.2). Tissue samples from the oviduct, uterus, cervix and vagina were collected from both groups for frozen and paraffin embedded sections (Location diagram below; Chapter 2 section 2.2.3.2). In addition, tissue



Location diagram. Examination sites of the reproductive tract of female rabbits. Sites from which tissue samples were collected lie between the parallel lines on the diagram.

samples of the small intestine and popliteal lymph nodes were collected from the normal rabbits for use as positive controls in immunohistochemistry. Monoclonal antibodies to rabbit CD45, CD43, the T-cell antigen KEN5, CD4, Ig μ chain, MHC class II (Chapter 2 Table 2-1) and polyclonal antibodies to rabbit IgA and IgG (Chapter 2, Table 2-2) were used to label leucocytes, T cells, B cells, APC, and plasma cells on

frozen sections of the reproductive tract using immunofluorescent methods (Chapter 2 section 2.4). For monoclonal antibody labelling, normal mouse ascites fluid (MAF) was used as a negative control for each assay. For polyclonal antibody staining, normal goat serum (Nordic) was used as a negative control for each staining. The slides were viewed using confocal microscopy and digital images were collected (Chapter 2 section 2.4.4). Paraffin embedded sections of the reproductive tract were prepared and stained for histological examination (Chapter 2 section 2.2.3.4). These sections were viewed using light microscopy and photographed using standard techniques.

3.3 Results

3.3.1 The effect of ovulation on the mucosal structure of the reproductive tract

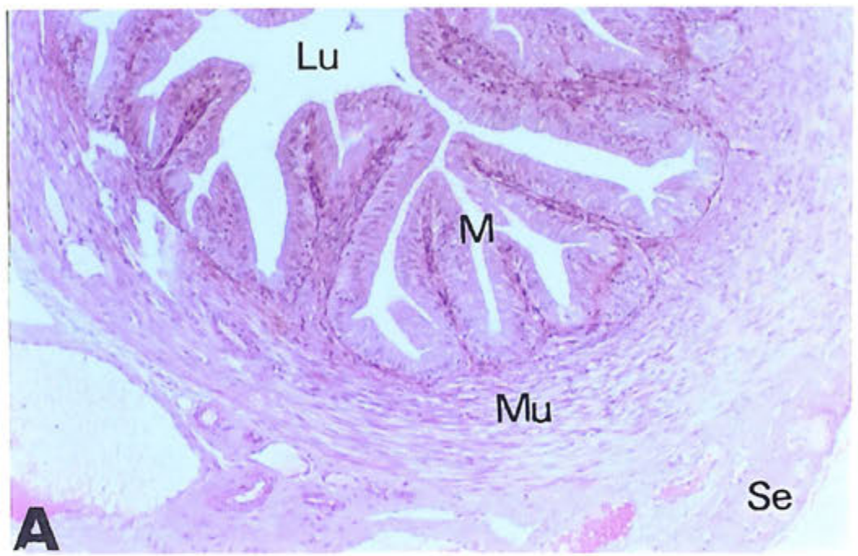
Induction of ovulation with hCG treatment resulted in a substantial increase in the size of the uterus, cervix and vagina. The mucosa of the uterus and vagina expanded 2-5 fold.

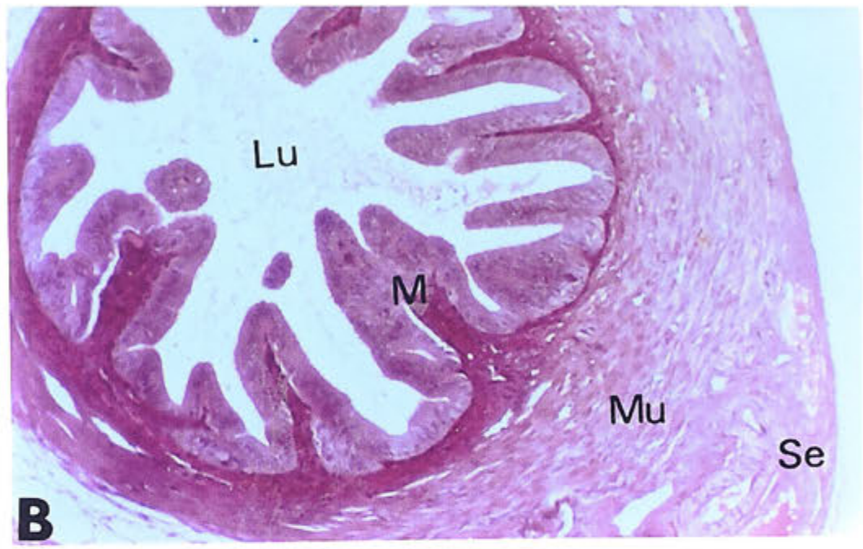
The histological structure of the mucosa at the examined sites (location diagram) of the oviduct, uterus, cervix, and vagina from normal and ovulated rabbits are shown in Figures 3-1.1, 3-1.2, 3-2.1, and 3-2.2. The epithelial cells of the luminal epithelium at those sites in most cases formed a simple columnar epithelium, the thickness and height of which varied from region to region (Figure 3-1.2, 3-2.2). However, in the ovulated uterus and cervix, the epithelium became pseudo-stratified columnar (Figure 3-1.2d, 3-2.2b). In addition, after induction of ovulation, the lamina propria was expanded (Figure 3-1.1, 3-2.1) and the epithelia became thicker in the uterus, cervix, and vagina, compared with those of normal rabbits (Figure 3-1.2, 3-2.2). In the cervix, the blood and lymphatic vessels in the lamina propria were enlarged and the epithelial cell layer was thickened compared with normal rabbits (Figure 3-2.2). In the oviduct, the cell density in the lamina propria increased after induction of ovulation (Figure 3-1.1, 3.1.2). These observations show that induction of ovulation has physically changed the structure of the mucosa in the reproductive tract.

Figure notes are on the other side

Figure 3- 1. 1. The mucosal structure of the oviduct and uterus from normal and ovulated rabbits. A is oviduct from a normal rabbit. B is oviduct from an ovulated rabbit. C is uterus from a normal rabbit and D is uterus from an ovulated rabbit. All are 100x magnification.

The rabbit oviduct (A and B) consists of three layers, from the lumen (Lu): mucosa (M), muscular layer (Mu) and serosa (Se). The mucosa includes the luminal epithelium and lamina propria. The two histological layers closest to the lumen (Lu) of the uterus (C and D) are the endometrium (Ed, including epithelium and stroma) and myometrium (My).


Lu
M
Mu
Se
A


Lu
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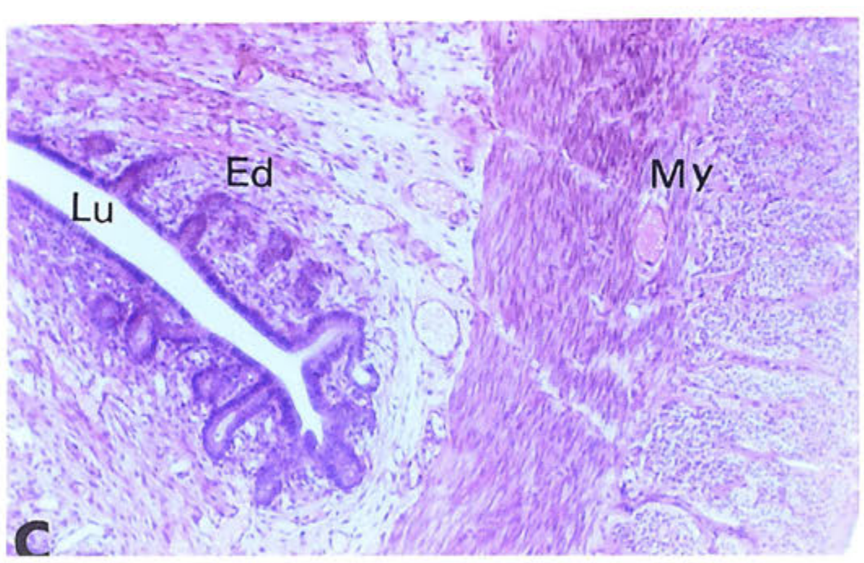
Ed
Lu
My
C

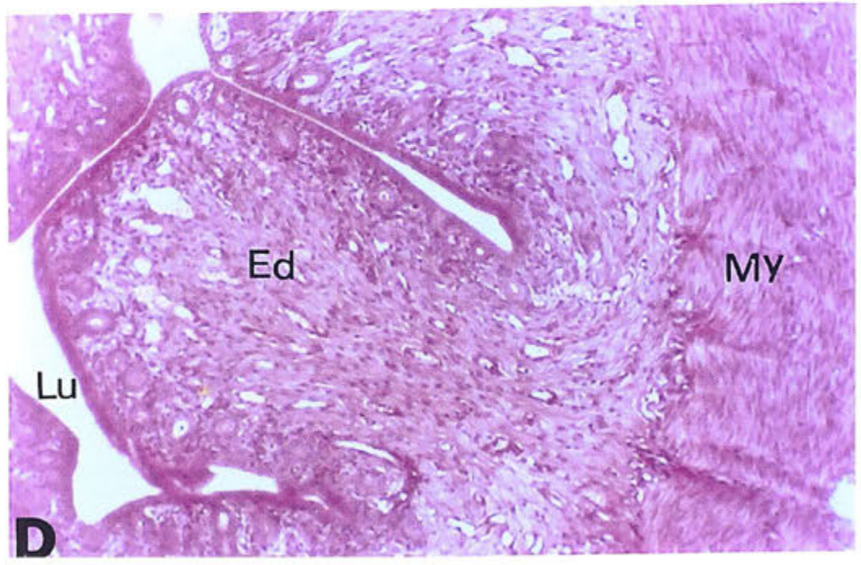
Ed
My
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Figure notes are on the other side

Figure 3-1. 2. The luminal epithelium of the oviduct and uterus from normal and ovulated rabbits. a is oviduct from a normal rabbit. b is oviduct from an ovulated rabbit. c is uterus from a normal rabbit and d is uterus from an ovulated rabbit. All are 400x magnification.

The luminal epithelia of normal (a) and ovulated (b) rabbit oviducts are typical simple columnar ciliated epithelium. The luminal epithelium of the uterus from normal rabbits (c) is also single columnar ciliated epithelium though the epithelial cells are thinner and shorter than those in the oviduct. Glands are also present in the normal uterine stroma (c, G). Lp: lamina propria; Ep: luminal epithelium; St: stroma.

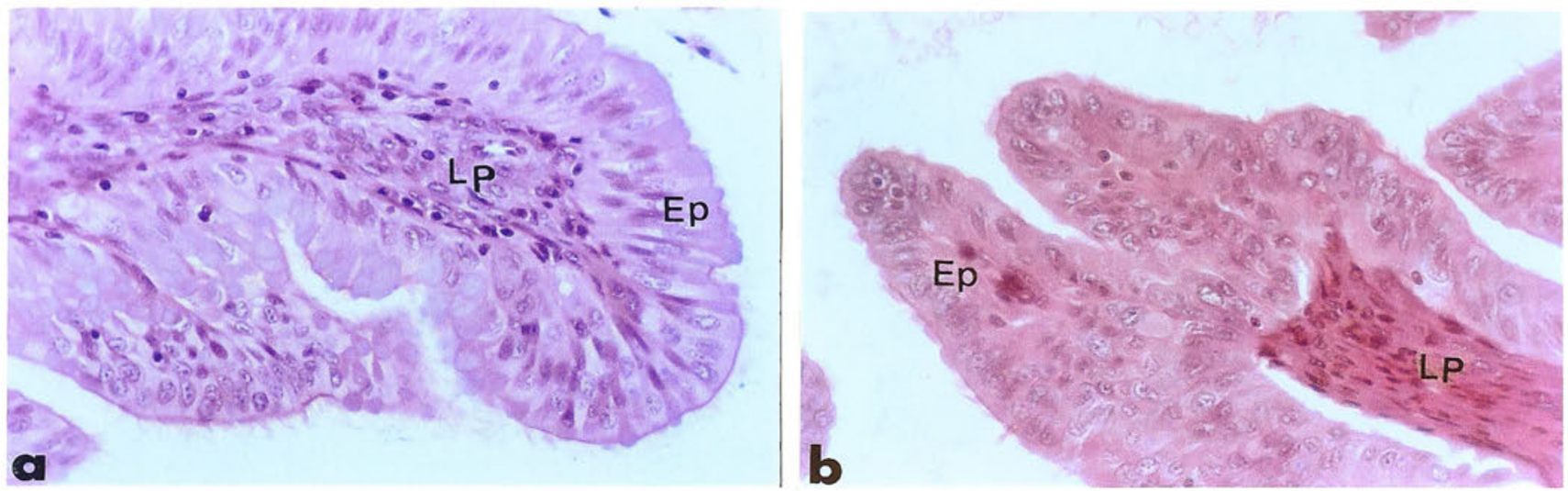
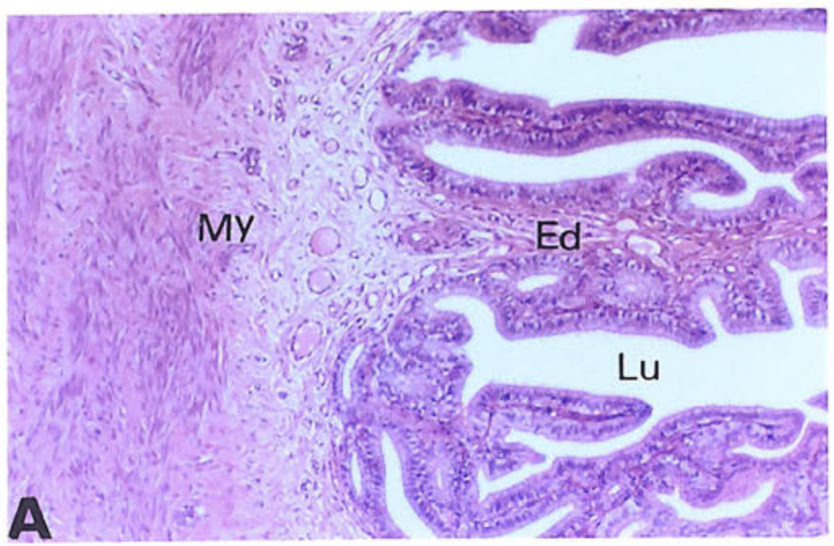

LP
Ep
a
Ep
LP
b
Ep
G
St
c
Ep
St
d

Figure notes are on the other side

Figure 3- 2. 1. The mucosal structure of the cervix and vagina from normal and ovulated rabbits. A is cervix from a normal rabbit. B is cervix from an ovulated rabbit. C is vagina from a normal rabbit and D is vagina from an ovulated rabbit. All are 100x magnification.

The two histological layers closest to the lumen (Lu) of the cervix (A and B) are the endometrium (Ed) and myometrium (My). The two inner histological layers for the vagina (C and D) are the mucosa (M, including the epithelium and lamina propria) and muscularis (Mus).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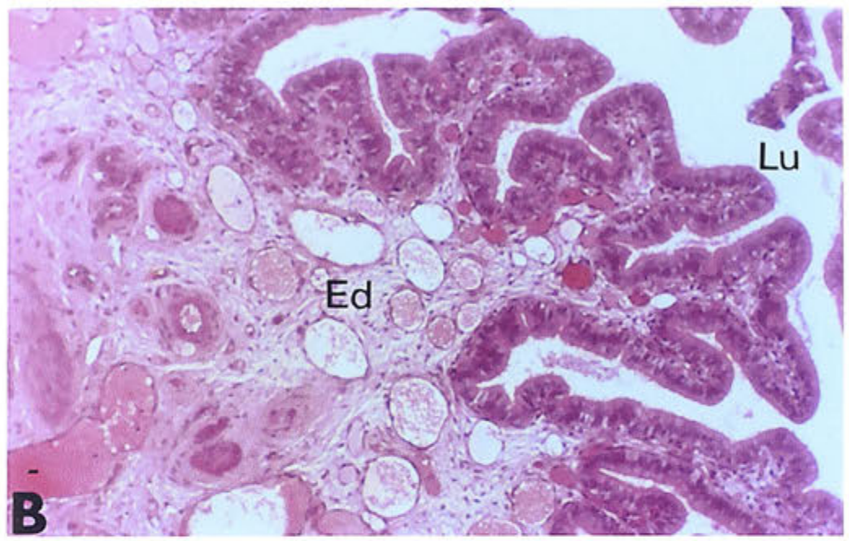
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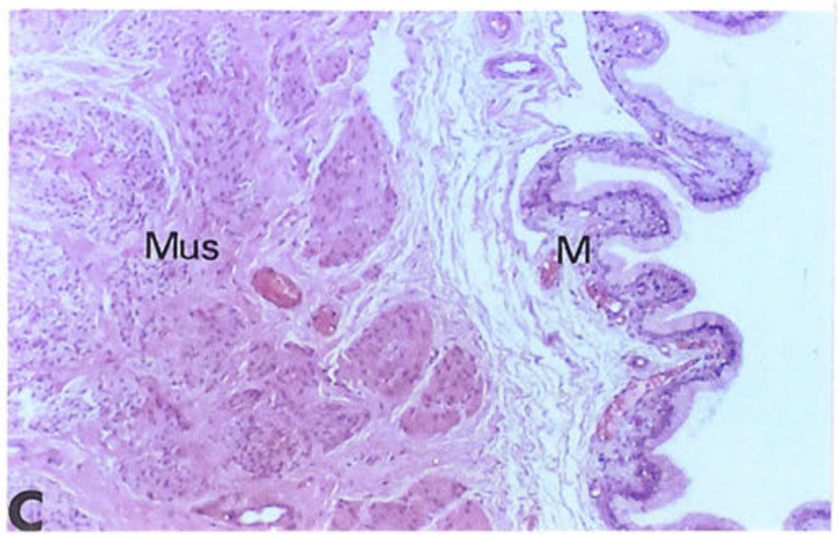
B


Mus
M
C

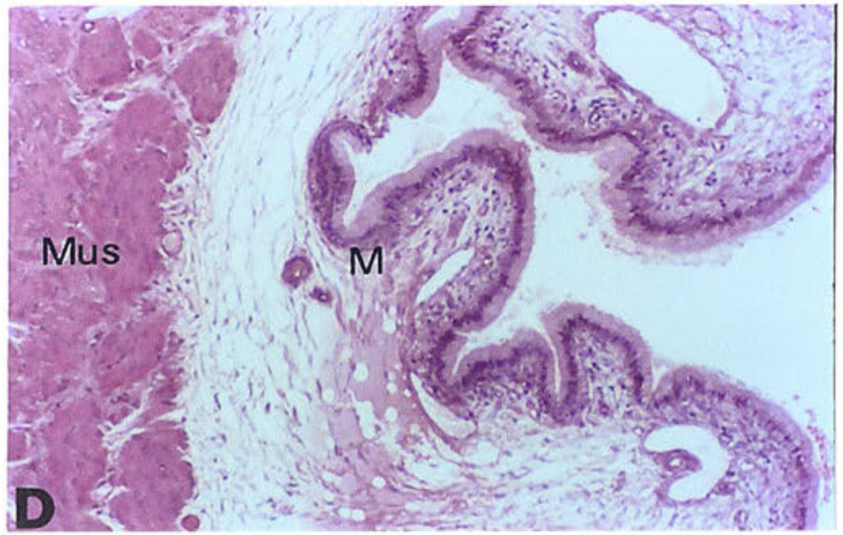
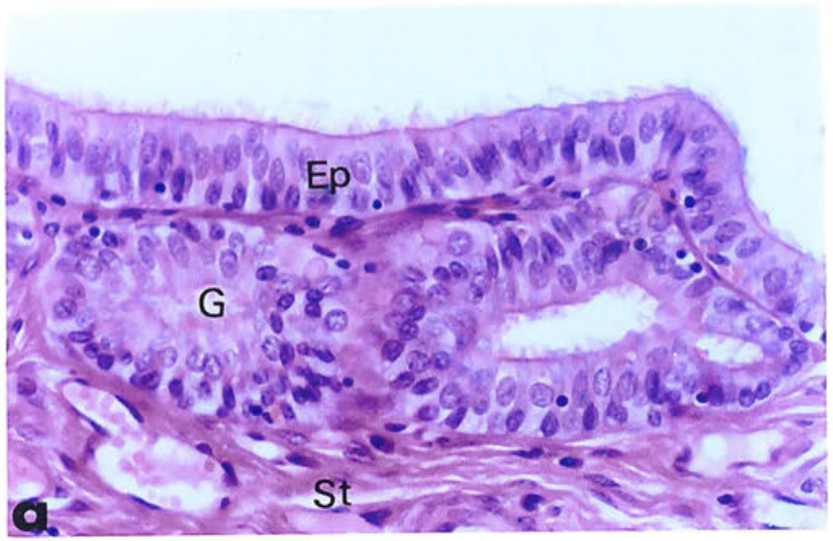

Mus
M
D

Figure notes are on the other side

Figure 3-2. 2. The luminal epithelium of the cervix and vagina from normal and ovulated rabbits. a is cervix from a normal rabbit. b is cervix from an ovulated rabbit. c is vagina from a normal rabbit and d is vagina from an ovulated rabbit. All are 400x magnification.

The luminal epithelia (Ep) of the cervix and vagina from normal (a and c) and ovulated (b and d) rabbits are simple columnar epithelium. However, the epithelium of the cervix is ciliated whereas the vaginal epithelium is not. Glands (G) can be seen in the stroma (St) of the cervix. LP: lamina propria.


Ep
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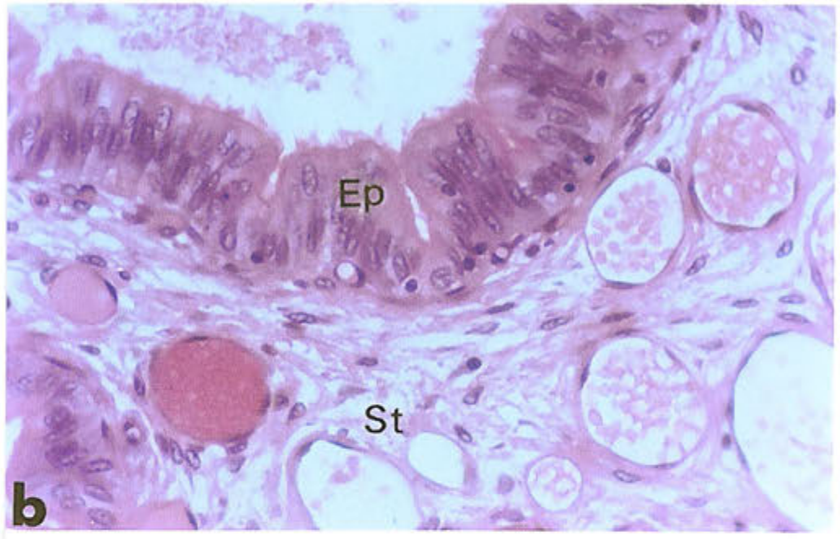
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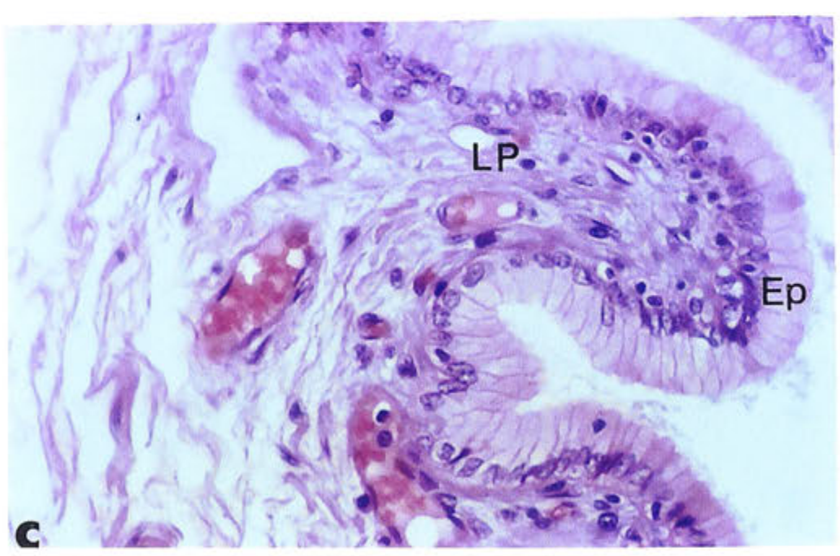
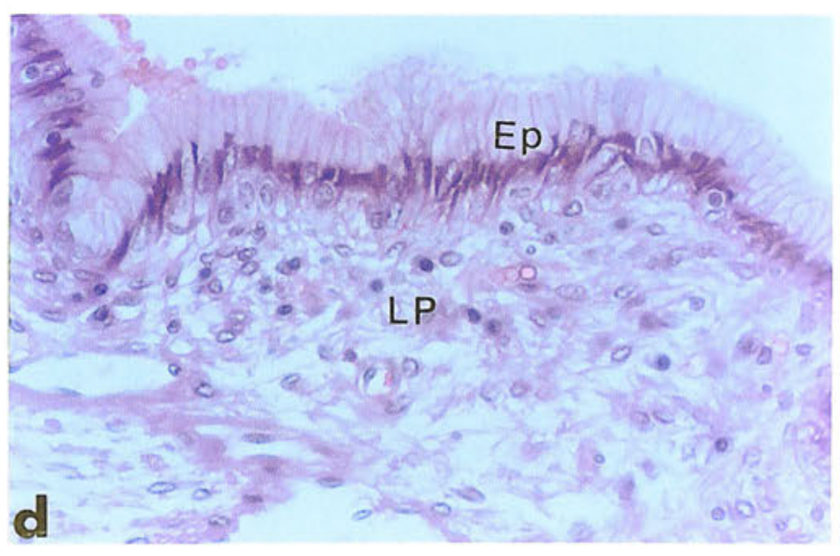

LP
Ep
c


Ep
LP
d

3.3.2 CD45 positive leucocytes in the reproductive tract

3.3.2.1 CD45+ cells in the normal reproductive tract

CD45 is a pan leucocyte marker. Anti-rabbit CD45 monoclonal antibody (Chapter 2 Table 2-1) binds to the leucocyte common antigen (LCA) and was employed to label leucocytes on frozen sections of the four reproductive tract regions using indirect immunofluorescence (Chapter 2 section 2.4.1). Normal mouse ascites fluid (MAF) was used as a negative control.

In most regions, numerous CD45+ cells were found in the luminal epithelium and in the area immediately beneath it but there were far fewer cells deep in the stroma or in the muscle layer (Figure 3-3). No positive cells were seen in any sections treated with MAF instead of monoclonal antibody (Figure 3-3). In addition, as positive and negative controls, frozen sections of popliteal lymph nodes were treated with anti-CD45 antibody or mouse ascites fluid. Whereas almost all of the cells were positive on the antibody–treated sections, no positive cells were seen on the sections treated with MAF (Figure 3-4). These results demonstrate the specificity of the CD45 antibody and the lack of background staining that occurred with this procedure.

The numbers of CD45+ cells in the mucosa of each region were counted using confocal microscopy (Chapter 2 section 2.4.4). Table 3-1 shows the mean of two or

Table 3- 1. CD45 positive cell (leucocyte) numbers in the mucosa of the normal reproductive tract

Rabbit No.	Oviduct	Uterus	Cervix	Vagina
361	72	28	120	194
437	90	17	128	130
440	133	19	115	156
456	62	12	63	98
Mean ± S.D	89 ± 31.4	19 ± 6.7*	107 ± 29.5	145 ± 40.6

The values are the cell number per mm^2 mucosa and represent the means of three counts in representative mucosal areas of each section. *: $P<0.01$ against other regions.

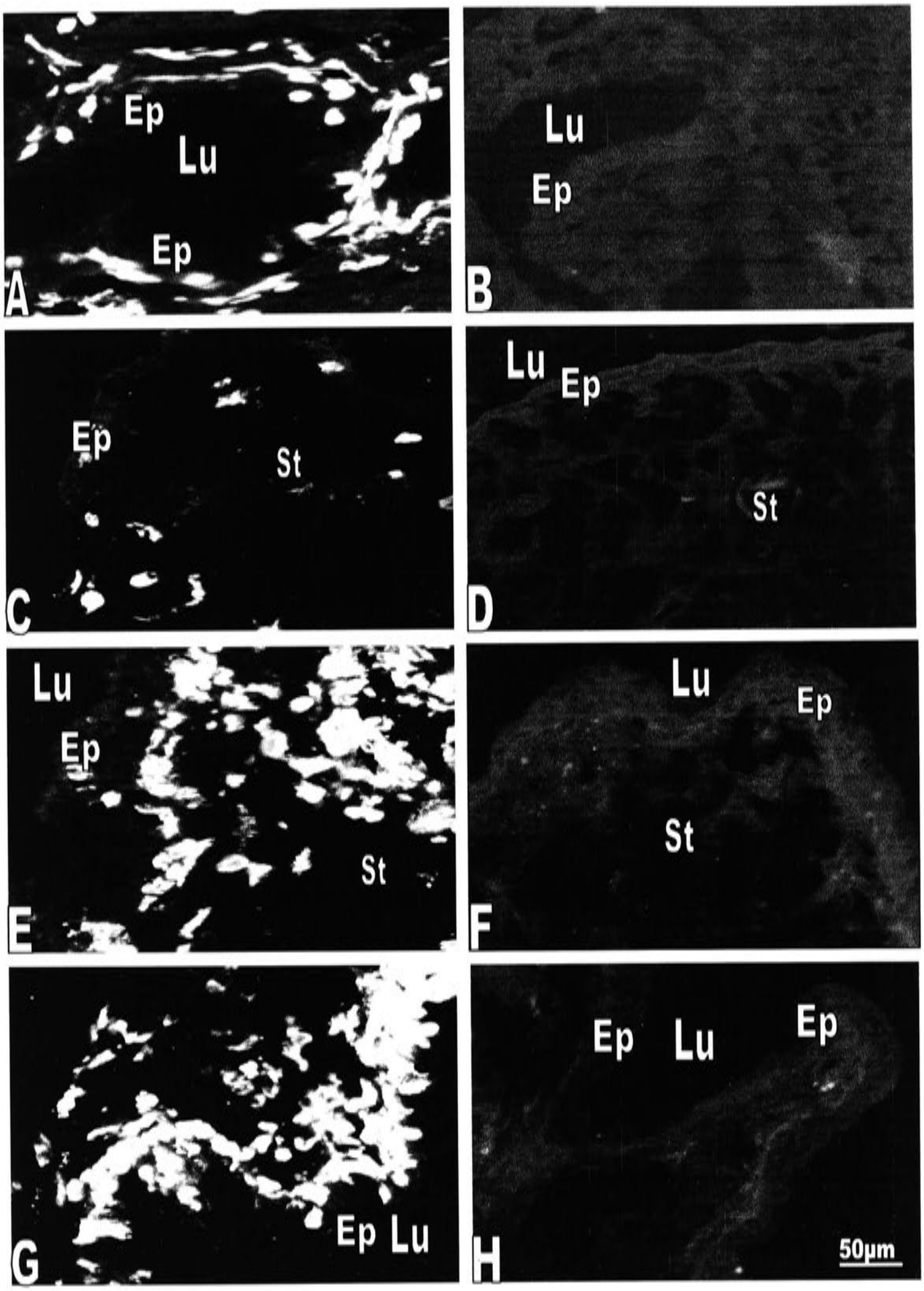


Figure 3-3. CD45 positive cells in the reproductive tract of normal female rabbits. The immunofluorescent images show CD45+ positive cells in the reproductive tract of normal female rabbits (four images on the left) and corresponding negative controls (four images on the right). A and B: oviduct; C and D: uterus; E and F: cervix, and G and H: vagina. Lu: lumen; Ep: luminal epithelium; St: stroma. All images are at the same magnification.

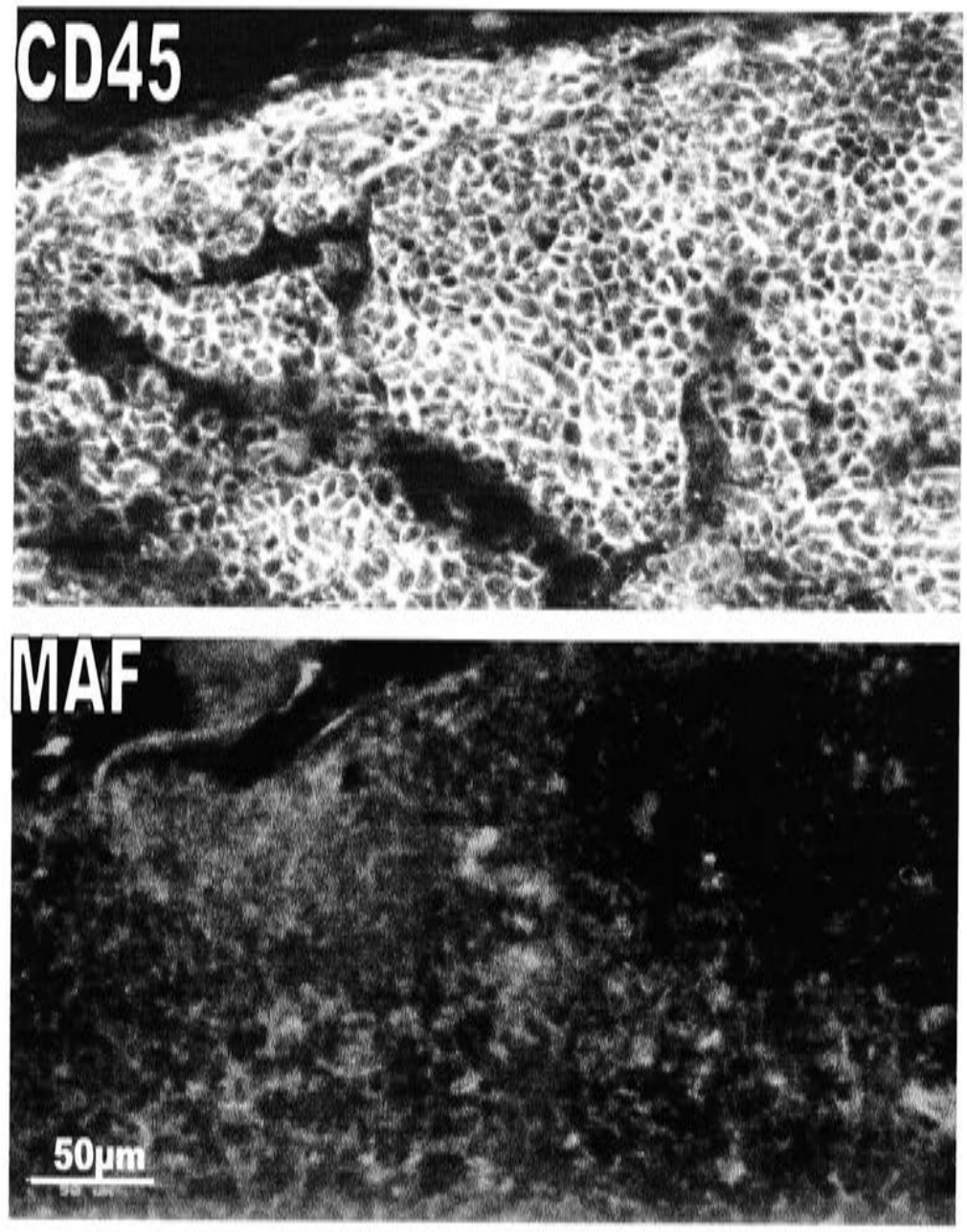


Figure 3-4. CD45 staining in rabbit popliteal lymph nodes. The immunofluorescent images show CD45 staining in popliteal lymph nodes as positive and negative controls for the staining. Positive control sections were treated with CD45 monoclonal antibody. Negative control sections were treated with MAF.

three counts in each of 2-3 representative mucosal areas. Where positive cells were not evenly distributed, and particularly in some cervical or vaginal sections with lymphocyte clusters, three counts were conducted in areas of low, medium, and high cell density. Although no significant differences were found between vagina, cervix and oviduct, significantly fewer CD45+ cells ($P<0.01$) were present in the endometrium of the uterus than in the mucosa of other regions (Table 3-1).

3.3.2.2 CD45+ cells in the ovulated reproductive tract

There was little change in the pattern of distribution of CD45+ cells in the four reproductive tract regions from ovulated rabbits compared with normal rabbits (Figure 3-5, Table 3-2). As in normal rabbits, significantly fewer positive cells were present in the uterus than in the other three regions (Table 3-2) and no differences in cell number were found between oviduct, cervix, and vagina. The cell number of each region in the hCG treated rabbits was not statistically different from that in normal rabbits (Table 3-1 and 2). However, there was an increase in the number of CD45+ cells around the blood vessels in the vaginal muscularis mucosa in 3 of 4 hCG treated rabbits compared with normal ones (Figure 3-6). For both normal and hCG treated rabbits, in some cervical and vaginal sections a large number of CD45+ cells were present directly beneath the epithelium forming cell clusters (Figure 3-6). Because these cells were located so close to the mucosal surface they may play an important role in local immunity.

Table 3- 2. CD45 positive cell (leucocyte) number in the mucosa of the ovulated reproductive tract

Rabbit No.	Oviduct	Uterus	Cervix	Vagina
398	71	51	139	149
351	137	10	110	135
453	0 [a]	41	206	133
441	99	27	69	123
Mean ± S.D	102 ± 33 (n=3)	32 ± 17.8*	131 ± 57.7	135 ± 10.7

The values are the cell number per mm^2 mucosa and represent the means of three counts in representative mucosal areas of each section. *: $P<0.01$ against vagina and $P<0.05$ against oviduct and cervix. a: no positive cells were found on the section, a repeat stain gave a similar result.

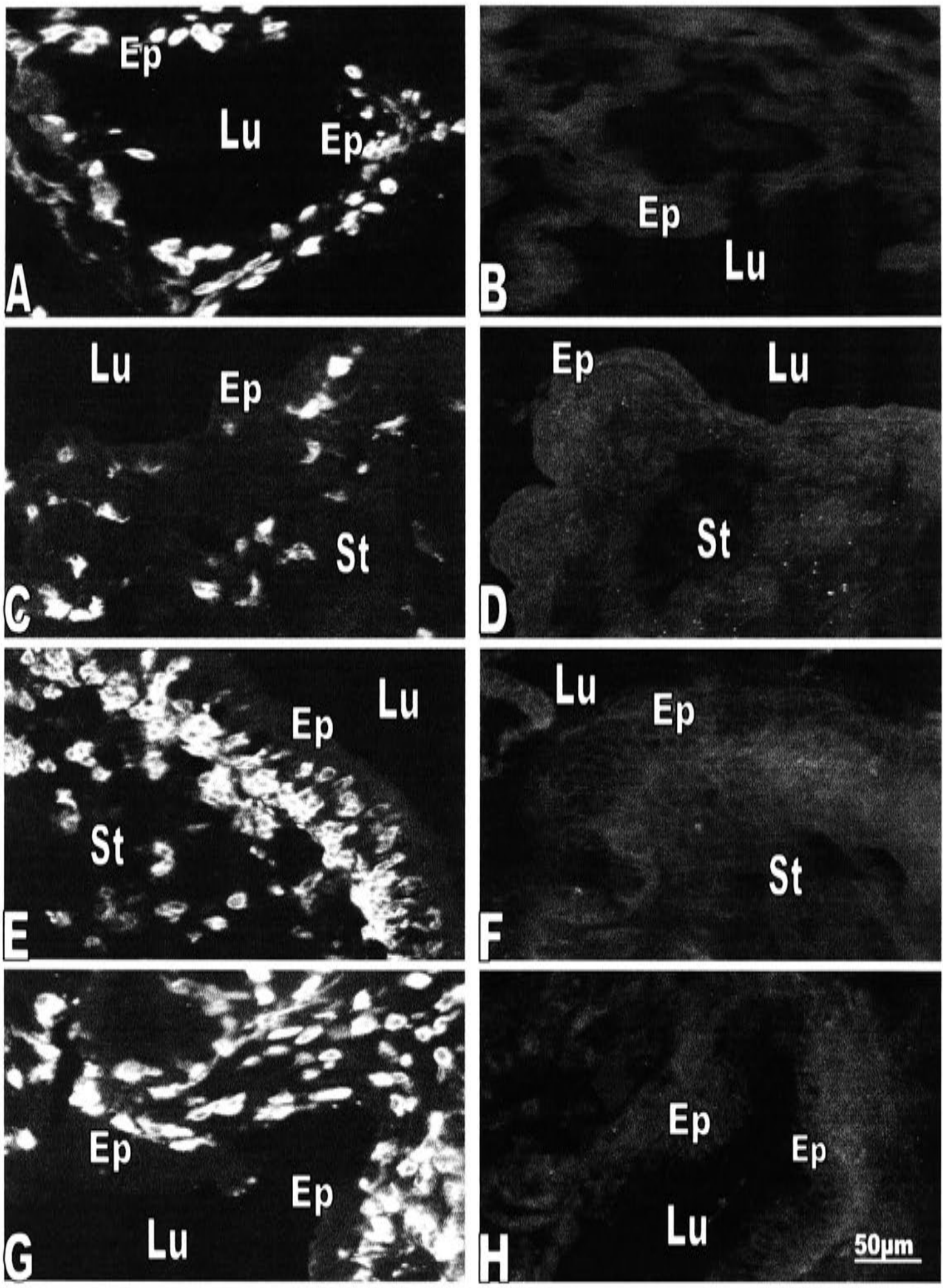


Figure 3-5. CD45 positive cells in the reproductive tract of ovulated rabbits. Immunofluorescent images show CD45+ positive cells in the reproductive tract of ovulated rabbits (four images on the left) with corresponding negative controls treated with MAF (four images on the right). A and B: oviduct; C and D: uterus; E and F: cervix, and G and H: vagina. Lu: lumen; Ep: luminal epithelium; St: stroma. All images are at the same magnification.

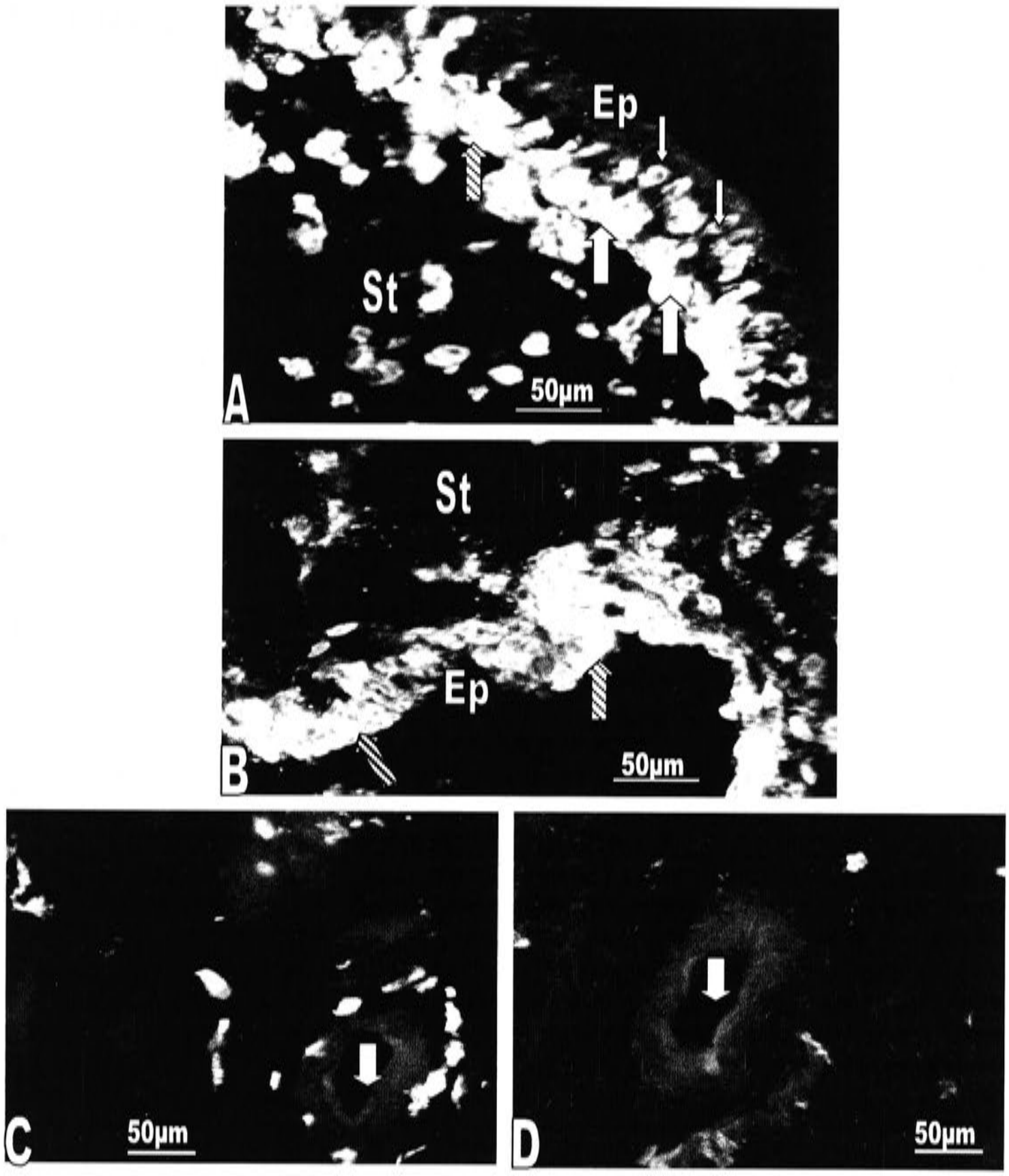


Figure 3-6. Sub-epithelial CD45+ cells in the cervix and CD45+ cells in the mucosa muscularis of the vagina after ovulation. Immunofluorescent images of CD45+ cells in the cervix of A) normal and B) ovulated rabbits highlight intra-epithelial CD45+ cells (narrow arrows) and sub-epithelial CD45+ cells (wide arrows). Some CD45+ cells form clusters (shaded arrow). C: Stromal tissue of ovulated rabbit vagina showing CD45+ cells around blood vessel (arrow). D: Stromal tissue of normal rabbit vagina showing scant CD45+ staining (arrow indicates blood vessel). Ep: luminal epithelium St: stroma.

3.3.3 CD43 positive cells in the reproductive tract

To classify T cells in the female rabbit reproductive tract, anti-rabbit CD43 monoclonal antibody L11/135 (Chapter 2, Table 2-1) was employed to stain T cells in frozen sections of the four reproductive tract regions. This monoclonal antibody was first described by Jackson *et al.* (1983) who reported that it specifically bound to rabbit T cells. A later study showed that L11/135 bound to the rabbit equivalent of human leukosialin/CD43, a molecule expressed on a wide variety of cells in the peripheral blood. However, in rabbits L11/135 binding was restricted to thymocytes and T cells with only a weak reaction to monocytes and macrophages (Wilkinson *et al.* 1992).

3.3.3.1 CD43+ cells in normal reproductive tract

Large numbers of CD43+ cells were present predominantly in the mucosal area of oviduct, cervix and vagina of the normal reproductive tract, with no significant differences in cell numbers between these regions (Figure 3-7, Table 3.3). The uterus had significantly fewer CD43+ cells ($P<0.01$). Few positive cells were present in the deep stroma or in the muscular layers of these regions. No positive cells were seen in any sections treated with MAF (not shown).

Table 3- 3. CD43+ T cell numbers in the mucosa of the normal reproductive tract

Rabbit No.	Oviduct	Uterus	Cervix	Vagina
361	41	21	104	146
437	41	17	113	96
440	47	6	83	123
456	0[a]	2	35	76
Mean ± S.D	43 ± 3.5 (n=3)	12 ± 7.8*	84 ± 34.8	110 ± 30.6

The values are the cell number per mm^2 mucosa and represent the means of three counts in representative mucosal areas of each section. *: $P<0.01$ against other regions. a: no positive cells were found on this section, the staining was repeated and a similar result was obtained.

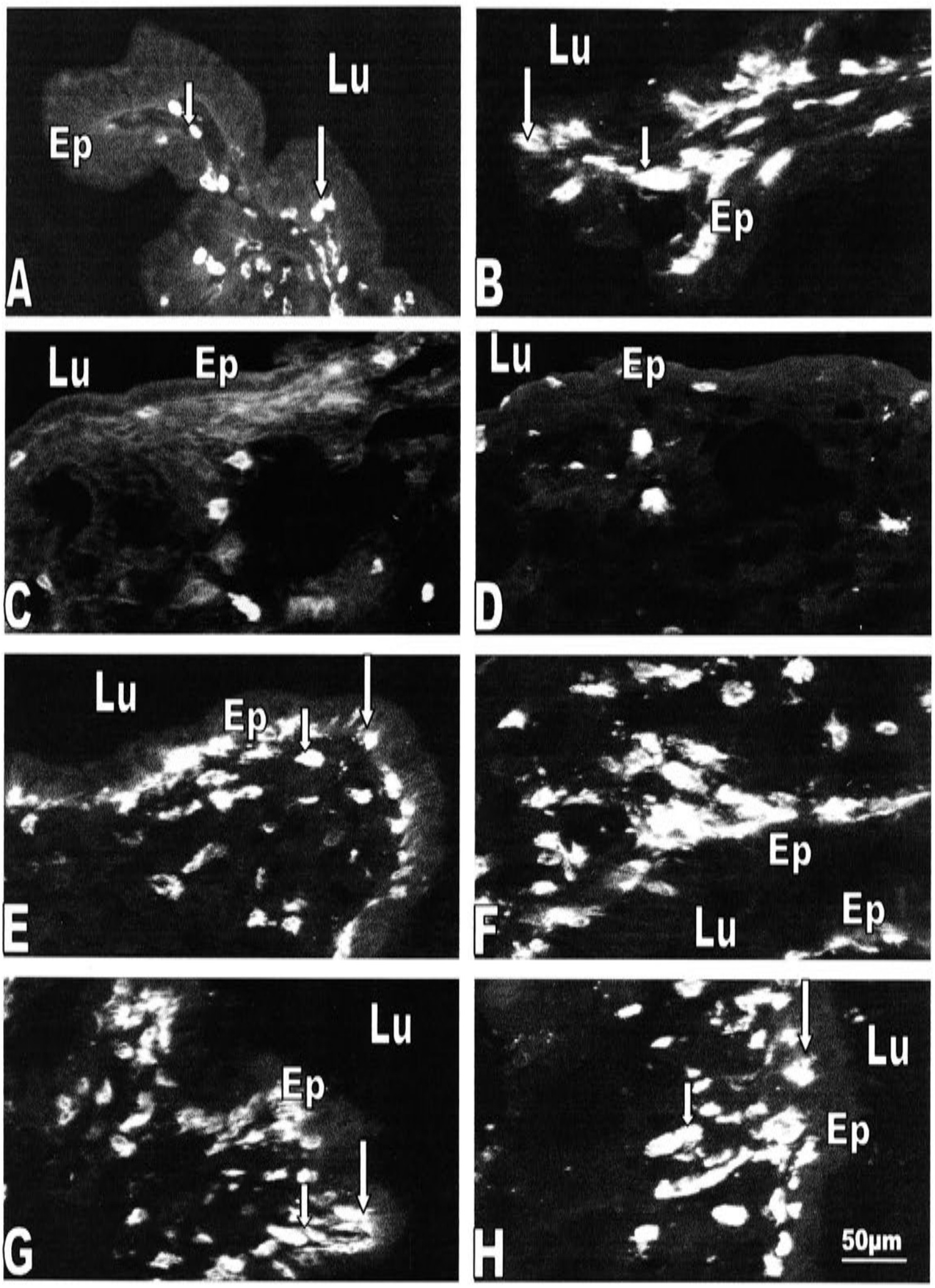


Figure 3-7. Immunofluorescent images of CD43+ cells in the reproductive tract of normal and ovulated female rabbits. The four images on the left are from normal rabbits and the four on the right are from ovulated rabbits. A, B: oviduct; C, D: uterus; E, F: cervix, and G, H: vagina. CD43+ cells were mainly located in the luminal epithelium (long arrows) and sub-epithelium (short arrows). All images are at the same magnification.

3.3.3.2 CD43+ cells in the ovulated reproductive tract

Induction of ovulation did not change the location or number of CD43+ cells in the four reproductive tract regions (Figure 3-7, Table 3-4). Like normal rabbits, CD43+ cells were mainly seen in the mucosal area of each region of the ovulated reproductive tract (Figure 3-7) and the cell number in each region was not statistically different from that in comparable regions from non-treated rabbits (Table 3-3 and 3-4). However, after ovulation the variability in cell number between individuals increased in most regions compared with normal rabbits (Table 3-4) and, as a consequence, the only significant difference in cell number in the different compartments was between the vagina and the uterus ($P<0.01$, Table 3-4).

Table 3- 4. CD43+ T cells in the ovulated reproductive tract

Rabbit No.	Oviduct	Uterus	Cervix	Vagina
398	8	32	102	80
351	77	2	40	77
453	22	25	127	110
441	72	9	29	101
Mean ±S.D	45 ± 34.9	17±13.9*	75±47.5	92±16.1

The values are the cell number per mm^2 mucosa and represent the means of three counts in representative mucosal areas of each section. *: $P<0.01$ against vagina.

As shown in Figure 3-7, the majority of CD43+ cells in the mucosa of the reproductive tract were located within the epithelium, or immediately beneath it, in both normal and ovulated rabbits. Because L11/135 mainly labels T cells in rabbits, those CD43+ cells located within the epithelium are presumably intra-epithelial lymphocytes (IEL, Figure 3-7 arrows). IEL have been suggested to play an important role in other species, in providing local immunity in the reproductive tract and/or in providing a suitable environment for implantation (Pace *et al.* 1991; Lohman *et al.* 1995).

3.3.3.3 The proportion of CD43+ cells to CD45+ cells

In humans, CD3+ cells (all T cells) form 30-60% of CD45+ leucocytes in the female reproductive tract (Givan *et al.* 1997). A similar trend was seen in rabbits. More CD45+ leucocytes than CD43+ cells were found in the female reproductive tract (Table 3-1, 2 and 3-3, 4; Figure 3-8) with CD43+ cells constituting 30-77% of CD45+ leucocytes (Table 3-5). For a direct visual comparison of CD43 and CD45 staining, adjacent sections of the four reproductive tract regions from a normal rabbit were labelled with CD43 and CD45; fewer CD43+ than CD45+ cells were seen in the comparable mucosal areas (Figure 3-8). After hCG treatment, the ratio of CD43+ cells to CD45+ cells decreased slightly but not significantly (Table 3-5).

Table 3- 5. The percentage of CD45+ cells that were also CD43+ in normal and ovulated reproductive tracts

Groups	Oviduct	Uterus	Cervix	Vagina
Normal	46 ± 11	56 ± 38	76 ± 15	77 ± 2
Ovulated	30 ± 23	44 ± 21	53 ± 17	69 ± 16

Notes: the values are mean ± S.D for four animals.

3.3.4 KEN-5 positive cells in the reproductive tract

Since monoclonal antibody L11/135 may also bind weakly to macrophages and monocytes in rabbits (Wilkinson *et al.* 1992), another monoclonal antibody, KEN-5, which was reported to bind to rabbit CD5 (Chapter 2 Table 2-1) was used to further classify T cells in the female rabbit reproductive tract. There were two monoclonal antibodies established against rabbit CD5. Clone R-CD5 was reported to mainly recognise rabbit B cells (Raman, 1992) whereas KEN-5 was reported to specifically recognise T cells without detection of any B cells in rabbits (Kotani *et al.* 1993). It is now considered that KEN-5 does not recognise CD5 but an unidentified T cell marker (Mage, 1998). Therefore the cells identified by KEN-5 are referred to as KEN-5 positive cells in the following text.

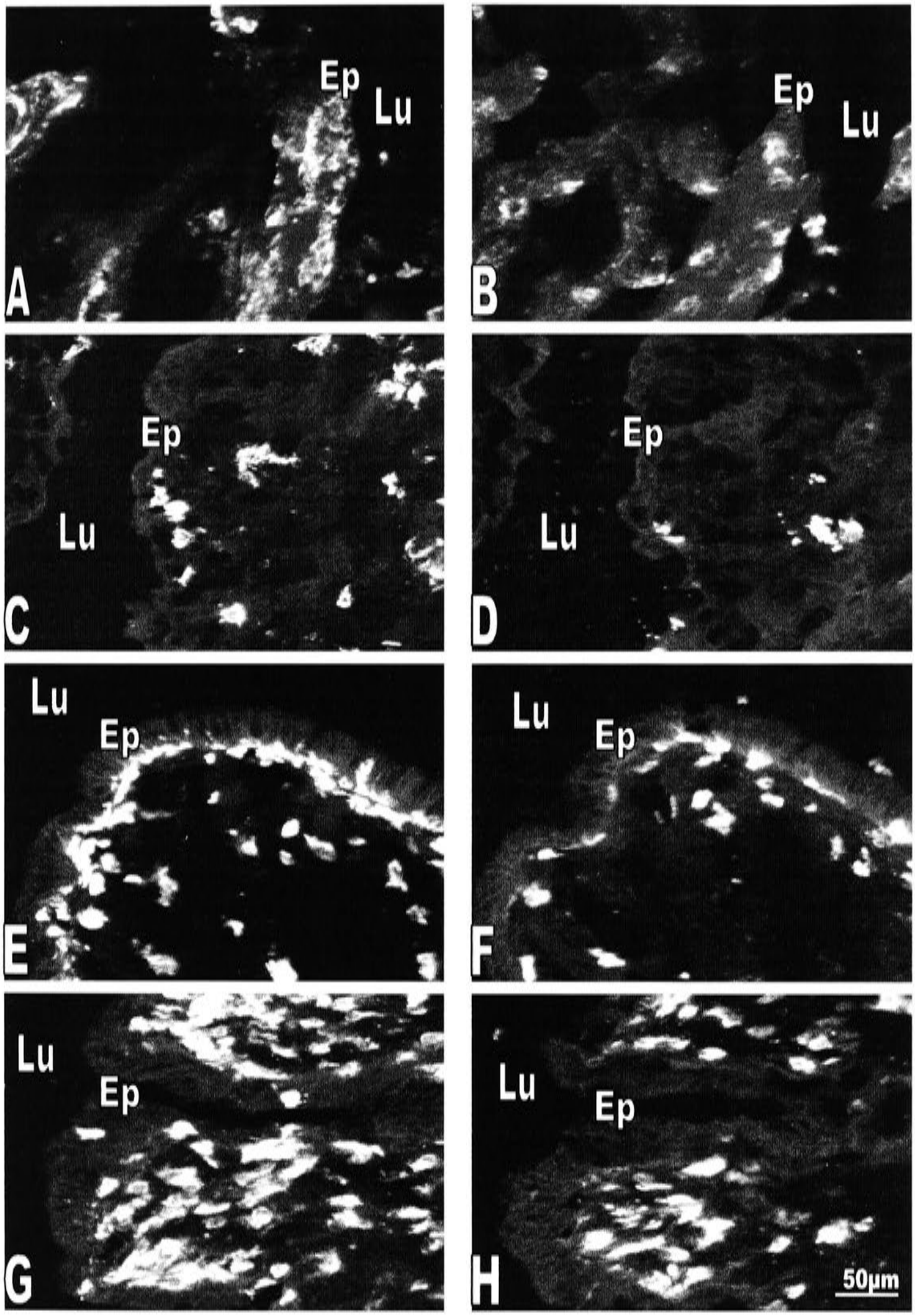


Figure 3-8. Comparison of CD45+ and CD43+ cells in the reproductive tract of normal rabbits. Immunofluorescent images show CD45+ (left panel) and CD43+ (right panel) cells in adjacent sections of oviduct (A, B), uterus (C, D), cervix (E, F), and vagina (G, H) from a normal rabbit. Lu: lumen; Ep: luminal epithelium. All images are at the same magnification.

3.3.4.1 KEN-5+ cells in normal reproductive tract

As was found for CD45+ and CD43+ cells, most KEN-5+ cells were located in the mucosal areas of each region and more positive cells were seen in the vagina and cervix than in the oviduct and uterus (Figure 3-9). However, compared with CD45+ or CD43+ cells, somewhat fewer KEN-5+ cells were present throughout the reproductive tract (Figure 3-9 cf. Figure 3-8). In the oviduct and uterus, fewer than 20 KEN-5+ cells/mm^2 were present in the epithelium or sub-epithelium. No cells were stained in the negative controls treated with normal MAF (not shown). Because of the difficulty of separating individual KEN-5+ cells in the epithelium of some cervical and vaginal sections no accurate count of positive cells in the mucosal area of these tissues could be conducted. However, it was estimated that more positive cells (~20-50 cells/mm^2) were seen in the epithelium and sub-epithelium of the cervix. Even more positive cells (~50-100 cells/mm^2) were found in the epithelium and sub-epithelium of the vagina.

3.3.4.2 KEN-5+ cells in the ovulated reproductive tract

After induction of ovulation, an increase in the number of KEN-5+ cells in the endometrium was seen in 3 of the 4 ovulated rabbits compared to normal rabbits (Figures 3-9 and 3-10). In tissues from ovulated animals, some positive cells formed clusters in the endometrium close to the epithelium (Figure 3-10), making identification of individual cells, and hence, counting of positive cells, impossible. Few changes in cell staining were seen in cervix or vagina after induction of ovulation (Figure 3-9) and no cells were stained in the negative controls treated with MAF.

3.3.5 Unsuccessful CD4 staining in the reproductive tract

A monoclonal antibody to rabbit CD4 (Chapter 2 Table 2-1) was also employed in an attempt to label CD4+ T cell (T helper cells). However, this antibody did not bind to any cells in the four reproductive tract regions and these data are therefore not shown. Other researchers attempting to stain tissue sections with this monoclonal antibody have experienced similar problems (P. J. Kerr, personal communication).

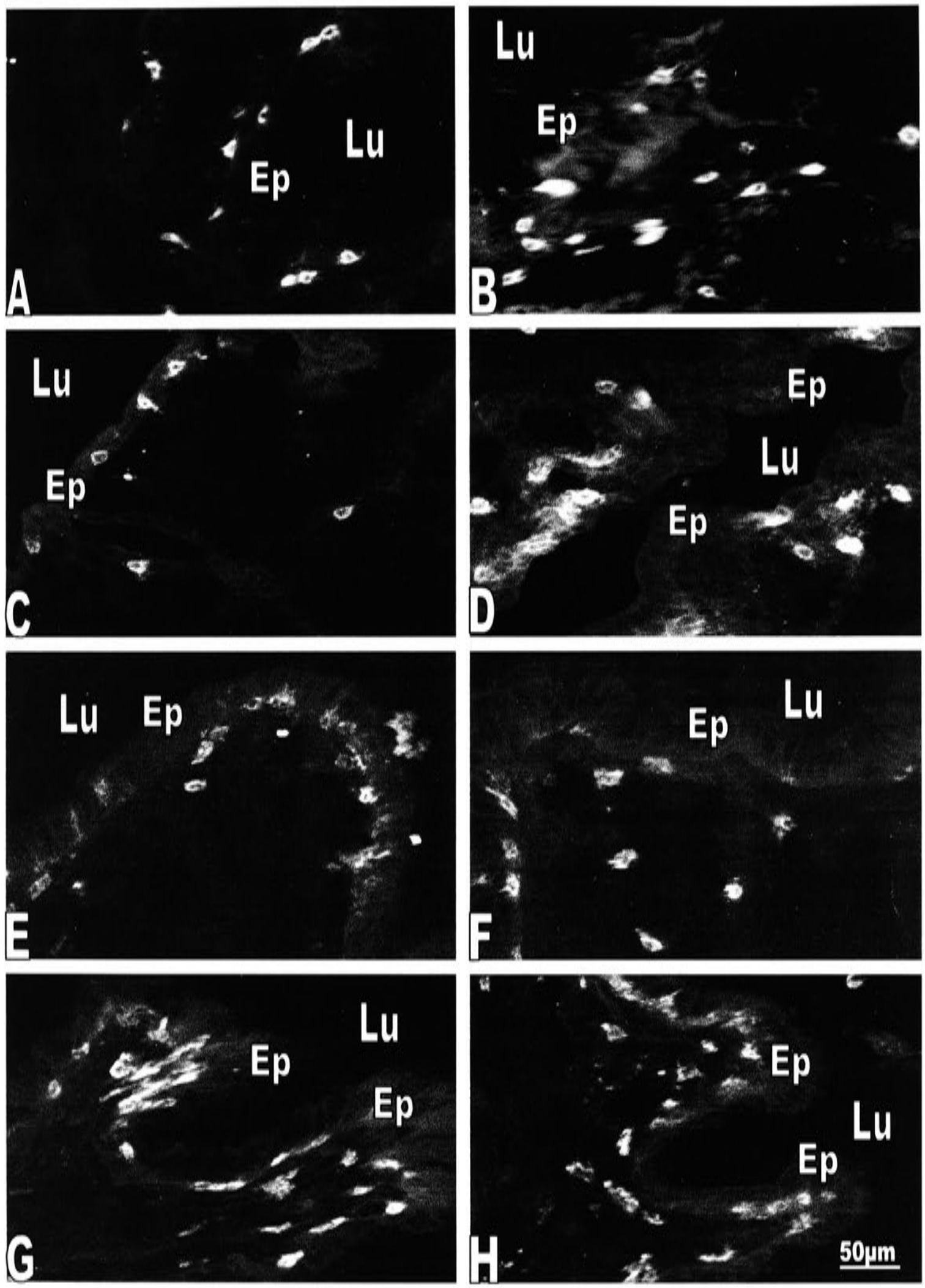


Figure 3-9. Immunofluorescent images of KEN-5+ cells in the female rabbit reproductive tract. The four images on the left are from normal rabbits and the four on the right are from ovulated rabbits. A, B: oviduct. C, D: uterus. E, F: cervix. G, H: vagina. Lu: lumen; Ep: luminal epithelium. All images are at the same magnification.

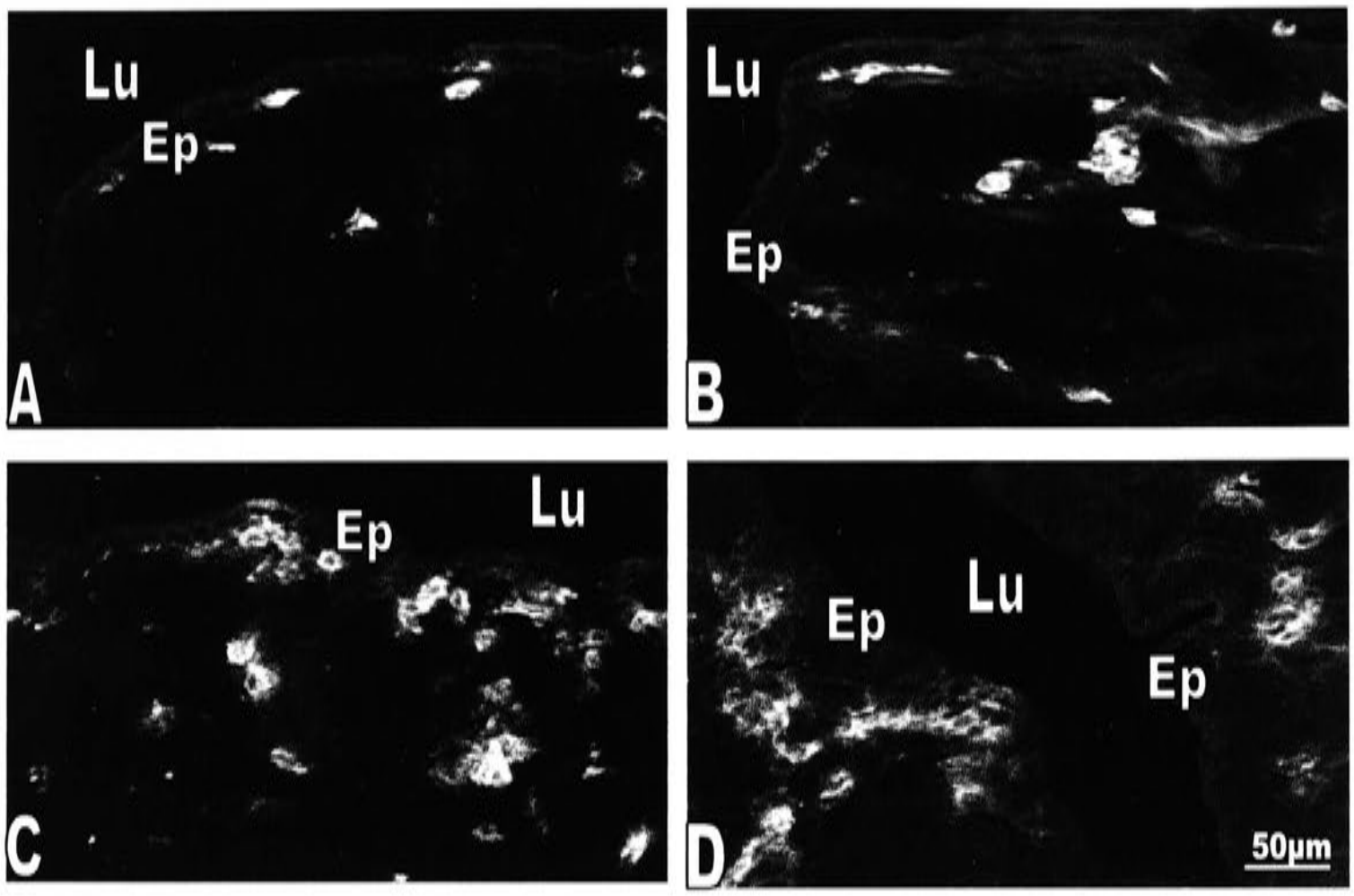


Figure 3-10. Immunofluorescent images of KEN-5+ cells in the endometrium of normal and ovulated rabbits. A: normal rabbit # 440. B: normal rabbit # 437 (normal rabbit #456 is shown in Figure 3-9c). C: ovulated rabbit # 453. D: ovulated rabbit # 441, (ovulated rabbit #351 is shown in Figure 3-9d). Lu: lumen; Ep: luminal epithelium. All images are at the same magnification.

3.3.6 MHC class II positive cells in the reproductive tract

MHC class II molecules (MHC II) expressed on APC play a crucial role in antigen presentation to CD4+ T cells by presenting processed peptides to the T-cell receptor (Chapter 1 section 1.5). In the reproductive tract of the female rat, MHC II expression was also shown to be important for antigen presentation in the tract (Wira and Rossoll, 1995b). Furthermore, in the female reproductive tract of rats (Wira and Rossoll, 1995; Wira *et al.* 2000) and humans (Fahey *et al.* 1999), both epithelial and stromal cells were shown to have the capabilities to present antigen. Rat uterine and vaginal epithelial or stromal cells were also MHC II positive (Kaushic *et al.* 1998). However, no such information is available for rabbits. To investigate this, a monoclonal antibody clone 2C4 (Chapter 2 Table 2-1) was employed to detect MHC II expression in frozen sections of the reproductive tract using immunofluorescence. The clone 2C4 was previously shown to recognise rabbit MHC Class II expressed on B cells and macrophages in lymphoid tissues (Lobel and Knight, 1984). In the reproductive tract, it may also recognise other MHC II positive cells such as Langerhans' cells or dendritic cells.

3.3.6.1 MHC II+ cells in normal reproductive tract

It is abundantly clear that MHC II+ cells are present in all mucosal compartments of the normal female rabbit reproductive tract (Fig 3-11). MHC II+ cells were predominantly present in the mucosal area of each region of the tract and fewer positive cells were present in the uterus than in the cervix, oviduct, and vagina (Figure 3-11). However, unlike leucocytes or CD43+ and KEN-5+ cells, the shape of MHC II+ cells varied from region to region. For instance, in the oviduct and uterus, most positive cells were still basically round-shaped (Figure 3-11) but in the cervix and vagina, most positive cells, especially those within the epithelium, were amorphous. The staining signals of positive cells within the epithelium were weak and the boundary of individual cells was difficult to identify (Figure 3-11) which made cell counting impossible.

In addition to the changes in cell shape, there were variations in the location of positive cells between regions. In the oviduct, positive cells were mainly located in the

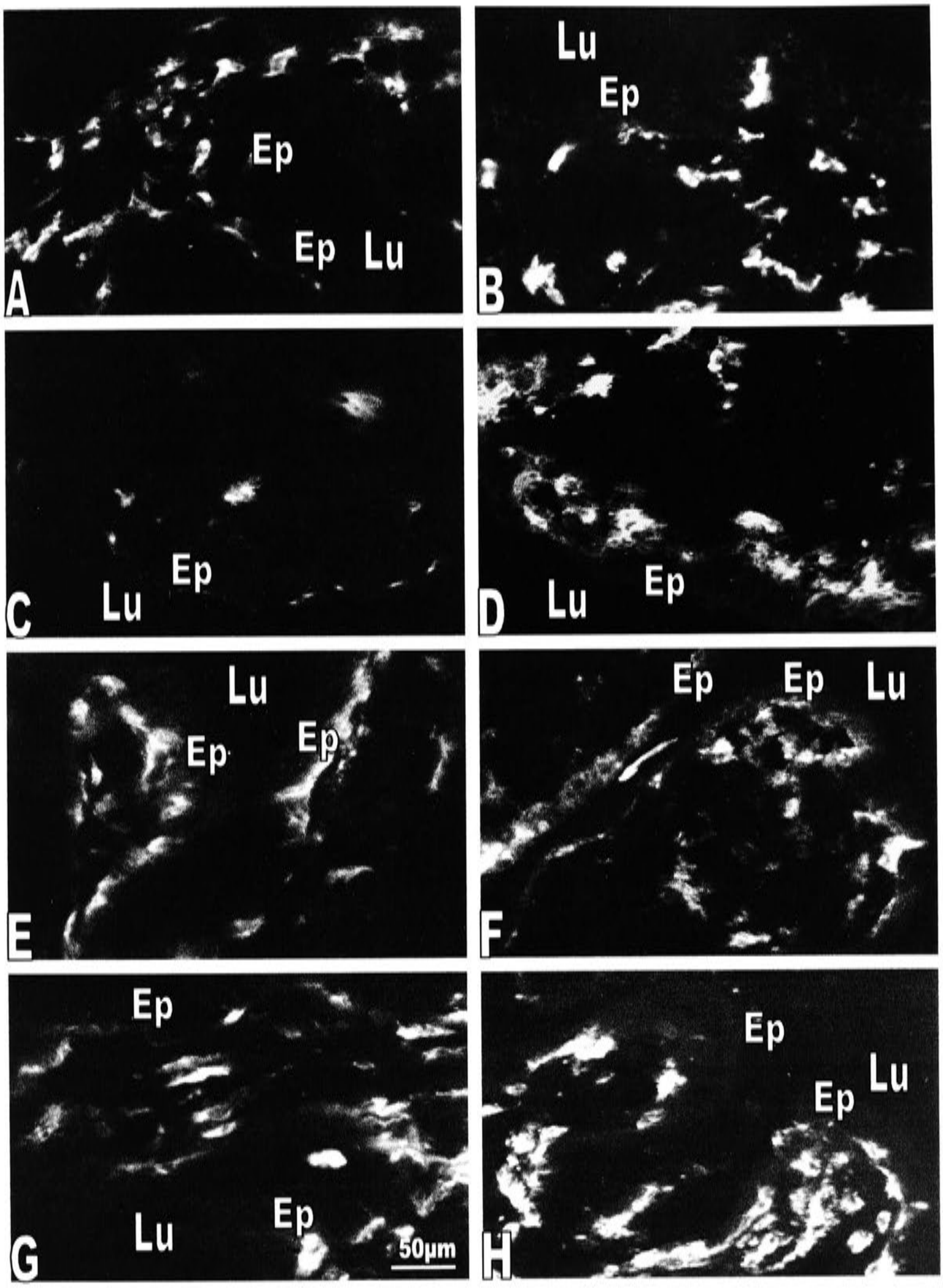


Figure 3-11. Immunofluorescent images of MHC-II+ cells in the female rabbit reproductive tract. The four images on the left are from normal rabbits; those on right are from ovulated rabbits. A, B: oviduct; C, D: uterus; E, F: cervix; G, H: vagina. Lu: lumen; Ep: luminal epithelium. All images are at the same magnification.

lamina propria and very few positive cells were seen in the epithelium (Figure 3-11A). However, in the cervix, positive cells were mainly located in, or immediately beneath the epithelium, with only a few positive cells in the lamina propria. In the vagina, though positive cells were present in the epithelium and immediately beneath it, they were also frequently seen in the lamina propria (Figure 3-11). When MAF was applied instead of the antibody no positive cells were seen in any sections (not shown).

3.3.6.2 MHC II+ cells in the ovulated reproductive tract

After induction of ovulation, there was an increase in the number of MHC II+ cells in the endometrium of 3/4 hCG injected rabbits compared with normal rabbits (Figure 3-11, 12) but there were no changes in the other three regions (Figure 3-11). In the uterus, after ovulation, some positive cells gathered closely together forming patches in the endometrium close to the luminal epithelium (Figure 3-12). Similar to CD43+ cells (Table 3-4), the extent of the variation in numbers of cells between animals increased after ovulation, especially in oviduct and cervix, suggesting that individual animals may have different responses to hCG treatment. No positive staining was seen in the negative controls treated with MAF (data not shown).

3.3.6.3 Double labelling with MHC class II and KEN-5 monoclonal antibodies

Double labelling with MHC class II and KEN-5 monoclonal antibodies results in the staining of three classes of cells. These are double-stained cells MHCII+/KEN5+ and two classes of single-stained cells, MHCII+/KEN5- and MHCII-/KEN5+. It appears that MHCII+/KEN5+ cells are T cells expressing MHC II. MHCII+/KEN5+ cells were found in all the regions of the reproductive tract but the cell number was not great (Figure 3-13). MHCII+/KEN5- cells were the dominant population in the cervix and vagina whereas MHCII-/KEN5+ cells were the majority in the oviduct (Figure 3-13). Far fewer positive cells were present in the uterus and these were mostly double positive cells.

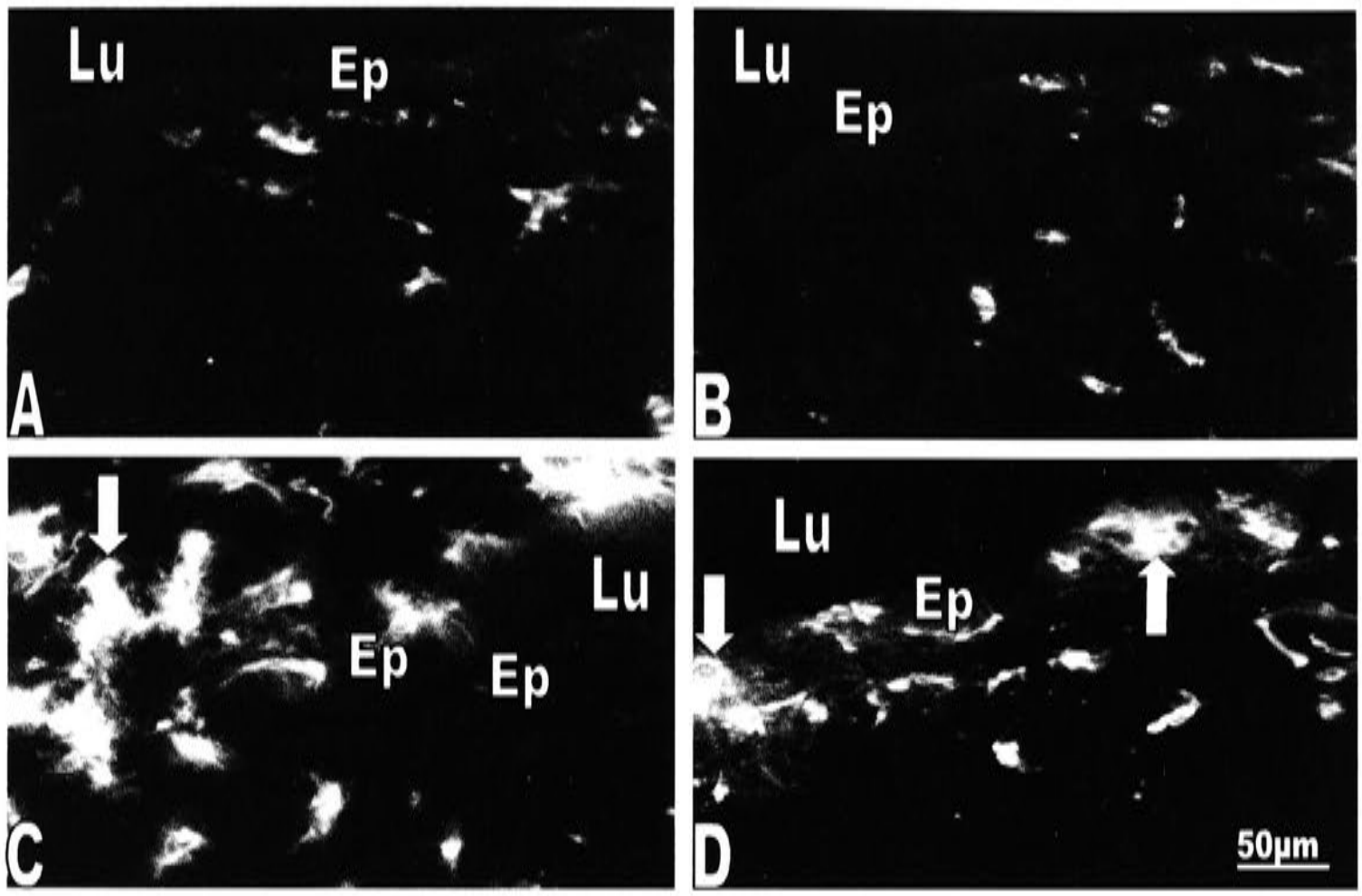


Figure 3-12. Comparison of MHC-II + cells in the endometrium of normal and ovulated rabbits. Immunofluorescent images of MHC-II+ cells in the uterus before and after ovulation. A, B are from two normal rabbits, different from the one in Figure 3-11(C). C, D are from two ovulated rabbits different from the one in Figure 3-11(D). In some cases, positive cells formed clusters (C, D, arrows) Lu: lumen; Ep: luminal epithelium. All images are at the same magnification.

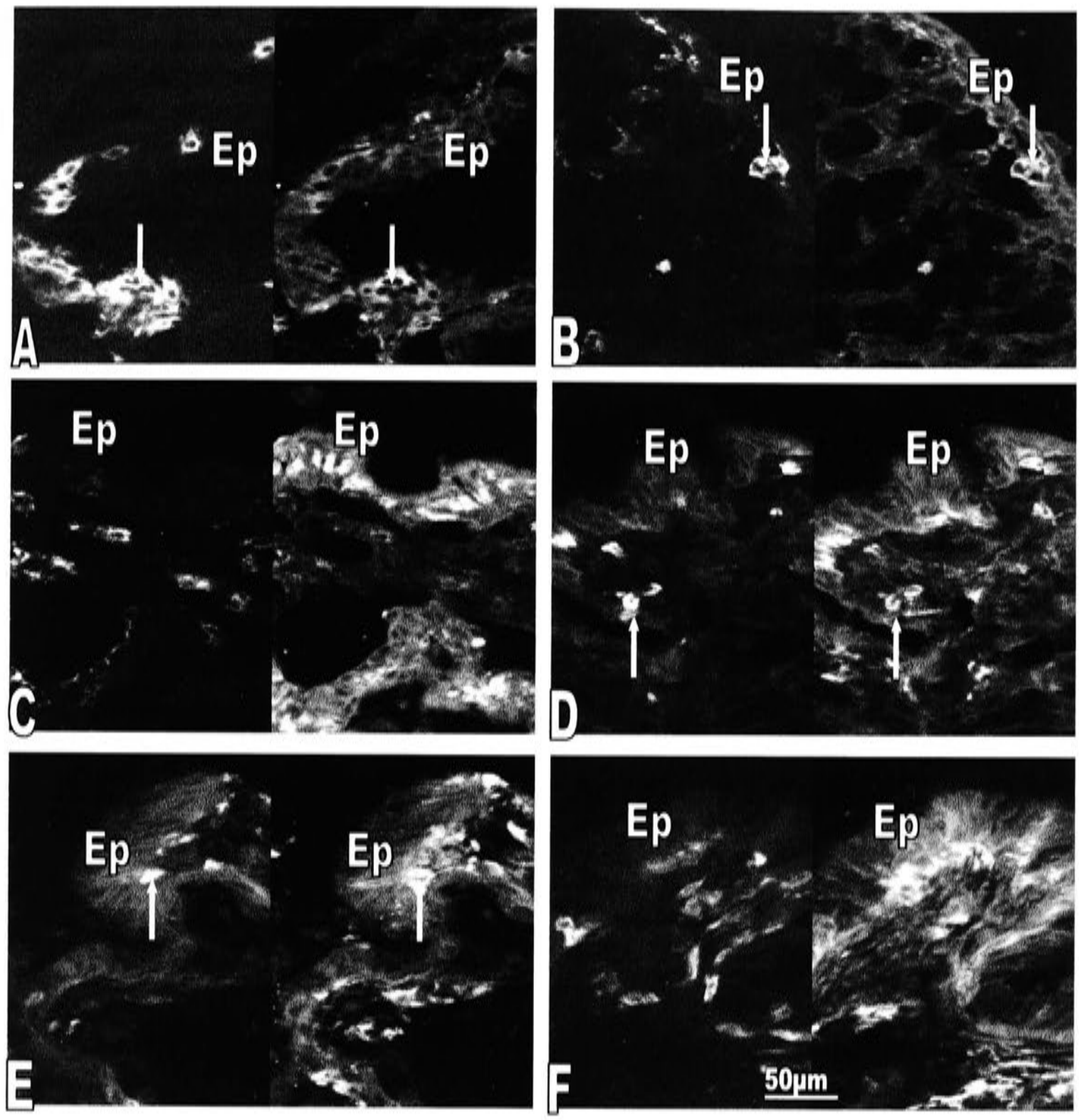


Figure 3-13. MHC II and KEN-5 double positive cells in the reproductive tract of normal female rabbit. Immunofluorescent images show double labelling with KEN-5 and MHC II monoclonal antibodies on frozen sections of oviduct (A), uterus (B), cervix (C, D) and vagina (E, F) of normal rabbits. Each image contains two parts; the left part shows KEN-5 staining and the right part shows MHC II staining on the same section. In the oviduct (A) more KEN-5+ than MHC II+ cells were present and KEN-5 staining was stronger than MHC II staining. In the uterus (B), most KEN-5+ cells were also MHC II positive (arrows). In the cervix and vagina, more MHC II+ cells than KEN-5+ cells are present in epithelium (C and E) or sub-epithelium (D and F) although there are some double positive cells (D and E, arrow). Ep: luminal epithelium. All images are at the same magnification.

3.3.7 Immunoglobulin μ-chain positive B cells in the reproductive tract

Antibody in the reproductive tract has two possible sources, transudation from serum or secretion by local plasma cells. The presence of localised, uncommitted B cells was examined by staining for Ig μ-chain to determine whether B cells were present in aggregates that could indicate a potential for local antigen presentation or the involvement of antibody responses. Normal MAF was used as a negative control.

3.3.7.1. Ig μ-chain positive cells in normal reproductive tract

Ig μ-chain positive B cells were sparse in the oviduct, cervix, and vagina and were rarely found in the uterus of the normal reproductive tract (Figure 3-14). The few positive cells were mainly in the lamina propria of the oviduct and vagina, or in the sub-epithelium and stroma of the cervix. No positive cells were seen in the negative control sections treated with MAF. As a positive control, frozen sections of popliteal lymph nodes were stained for Ig μ chain positive cells. These cells were common in the cortex of lymph nodes (Figure 3-15). In addition, when similar staining was applied to frozen sections of the vagina from a birth-experienced rabbit, positive cells were easily found in the mucosal area (Figure 3-15). These positive controls demonstrate that the monoclonal antibody is able to recognise positive cells in frozen sections and confirm the sparsity of Ig μ-chain positive cells in the normal reproductive tract.

3.3.7.2. Ig μ-chain positive cells in the ovulated reproductive tract

Induction of ovulation had no effect on B cell number or location within the reproductive tract. As with normal rabbits, no positive cells were found in the uterus and very few positive cells were present in the oviduct, cervix, or vagina (Figure 3-14). No positive cells were seen in the control sections treated with MAF.

3.3.8. IgA containing cells in the reproductive tract

Ig μ-chain bearing B cells were not commonly present in the female rabbit reproductive tract. However, the presence of IgA plasma cells may be more important

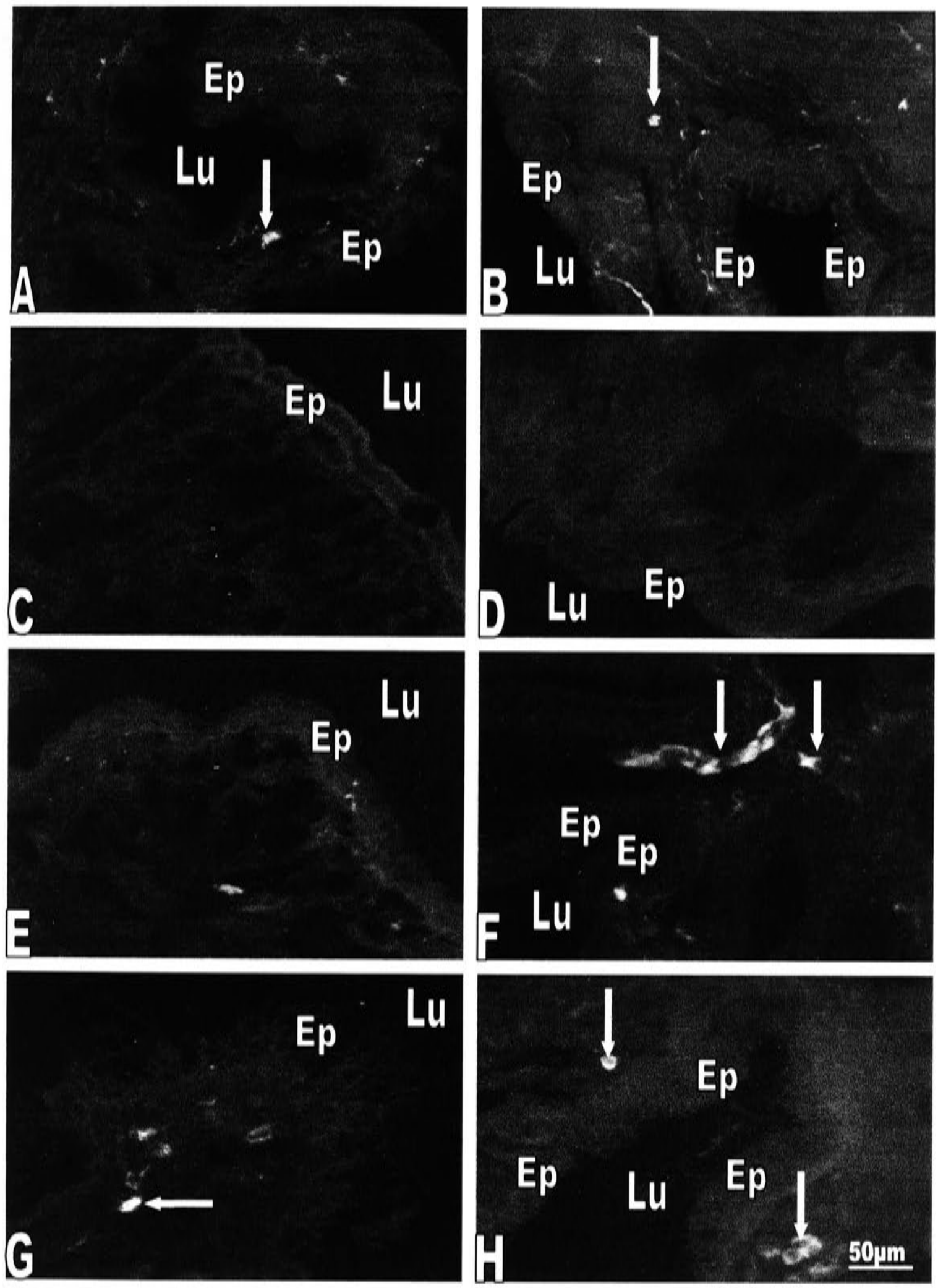


Figure 3-14. Immunoglobulin μ-chain positive cells in the female rabbit reproductive tract. Immunofluorescent images show rabbit Ig μ chain positive cells in the oviduct (A, B), uterus (C, D), cervix (E, F), and vagina (G, H) of the female rabbit. The four images on the left are from normal rabbits and the images on the right are from ovulated rabbits. Arrows indicate positive cells. Lu: lumen; Ep: luminal epithelium. All images are at the same magnification.

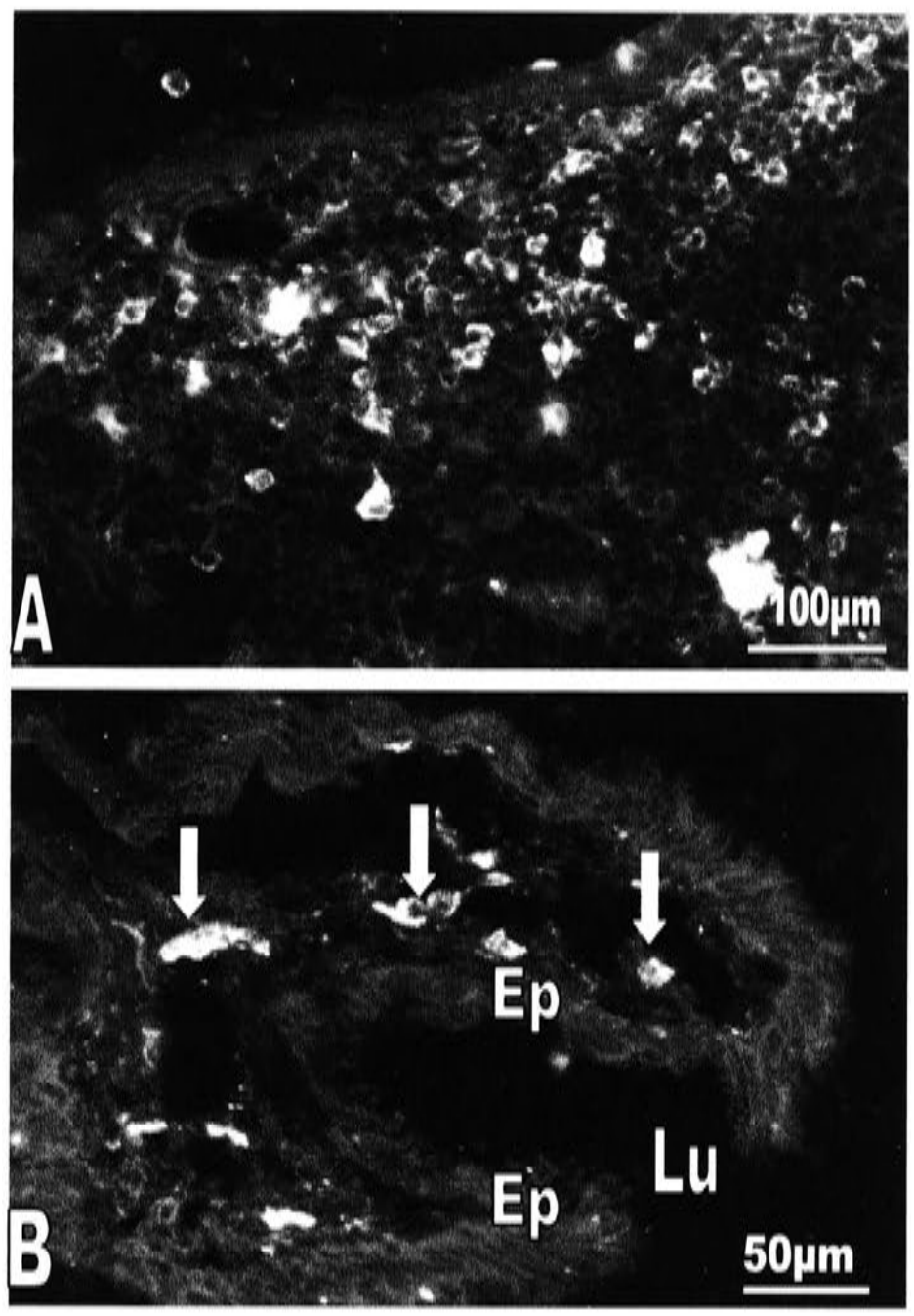


Figure 3-15. Ig μ chain positive cells in the popliteal lymph nodes and vagina. Immunofluorescent images of Ig μ chain staining of frozen sections of popliteal lymph nodes. A: cortex of the lymph nodes. B: vagina from a birth-experienced rabbit (arrows indicate positive cells). Lu: lumen; Ep: luminal epithelium.

for local mucosal immunity (Chapter 1 section 1.4.2). A previous study showed that a few IgA plasma cells were present in the endometrium of rabbits (Otsuki *et al.* 1990), but no information was available on IgA plasma cells in any other regions of the tract. To detect IgA producing cells, goat anti-rabbit IgA antibody (Chapter 2 Table 2-2) was employed to label IgA containing cells in frozen sections of the reproductive tract using indirect immunofluorescence (Chapter 2 section 2.4.1). Normal goat serum (Nordic) was used as negative controls.

3.3.8.1. IgA containing cells in normal reproductive tract

IgA positive cells were not commonly seen in any regions of the reproductive tract including the cervix and vagina and the IgA staining on the positive cells was usually weak (Figure 3-16). These weakly positive cells were mainly located in the lamina propria of the oviduct, the sub-epithelium or around the glands of the uterus, the sub-epithelium or deep stroma of the cervix, and sub-epithelium or lamina propria of the vagina. Apart from these positive cells, strong IgA staining was consistently seen along the apical area (Figure 3-16c), or sometimes both apical and basal areas of the luminal epithelium of the uterus. IgA staining was also seen in the basal areas of cervical epithelial cells or sometimes in a small part of the vaginal epithelium. In addition, in the uterus and vagina, IgA staining was seen on the internal epithelial surface of some glands (Figure 3-16). In contrast, no positive cells and no other staining were found in the negative controls treated with normal goat serum (Figure 3-16).

IgA staining was also examined in the small intestine as a positive control. Far more IgA positive (plasma) cells were seen in the lamina propria and sub-epithelium of the intestine (Figure 3-17), demonstrating that this antibody can specifically bind to IgA containing cells in frozen sections.

3.3.8.2.IgA containing cells in the ovulated reproductive tract

After induction of ovulation, no change in the number or location of IgA positive cells in the reproductive tract was seen and IgA staining along the surfaces of luminal and glandular epithelia of the uterus was still observed (Figure 3-18). Furthermore, IgA

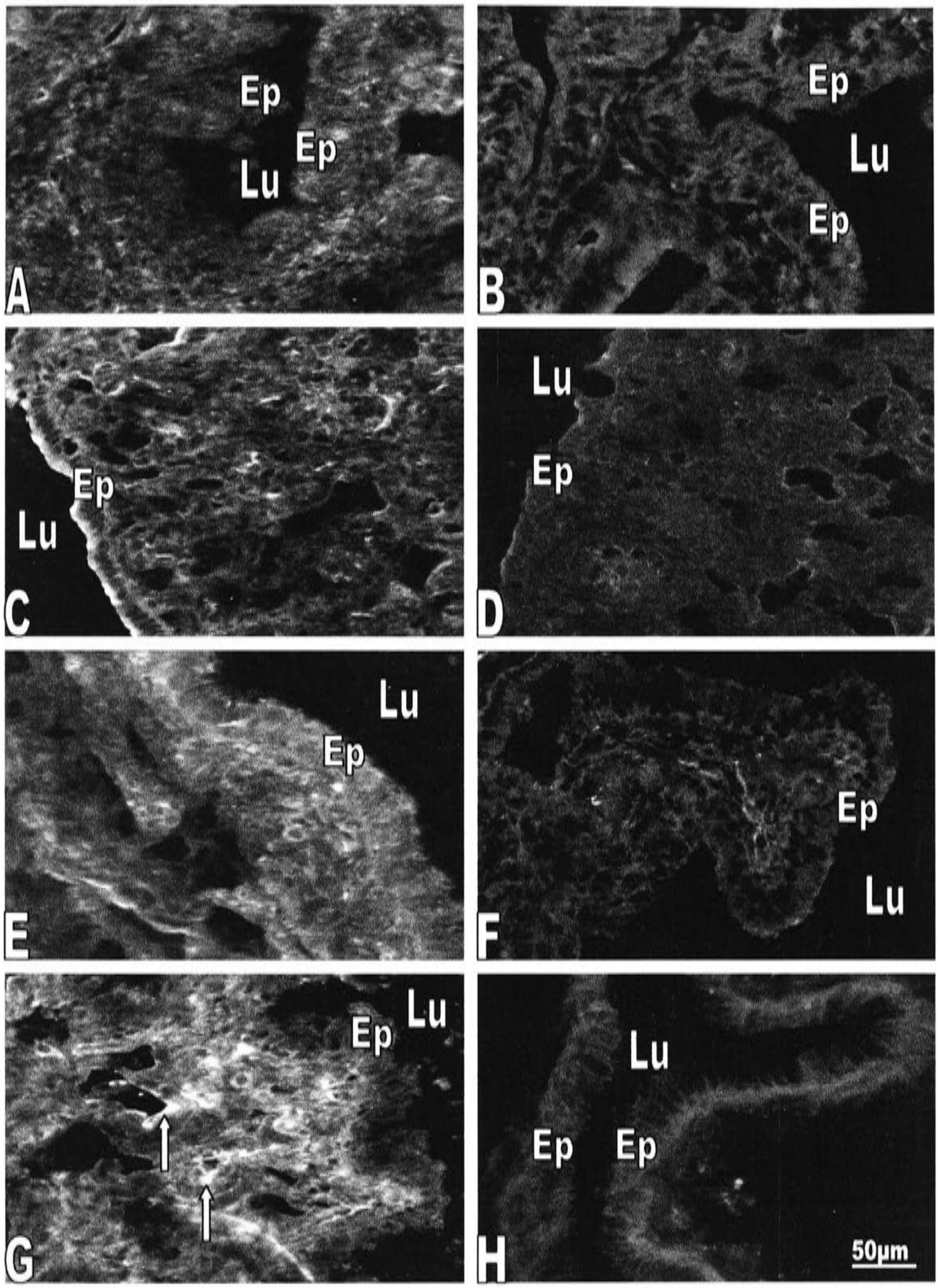


Figure 3-16. IgA staining in the reproductive tract of normal female rabbits. The four immunofluorescent images on the left show IgA staining in the normal rabbit reproductive tract and the four images on the right are the negative controls treated with normal goat serum. A and B: oviduct; C and D : uterus; E and F : cervix, and G and H : vagina. Note: strong IgA staining along the apical edge of the luminal epithelium of the uterus and on the epithelial surface of glands in the uterus and vagina (C and G, arrows). In the negative controls there was a little non-specific background in cervix (F) and vagina (H). Lu: lumen; Ep: epithelium. All images are at the same magnification.

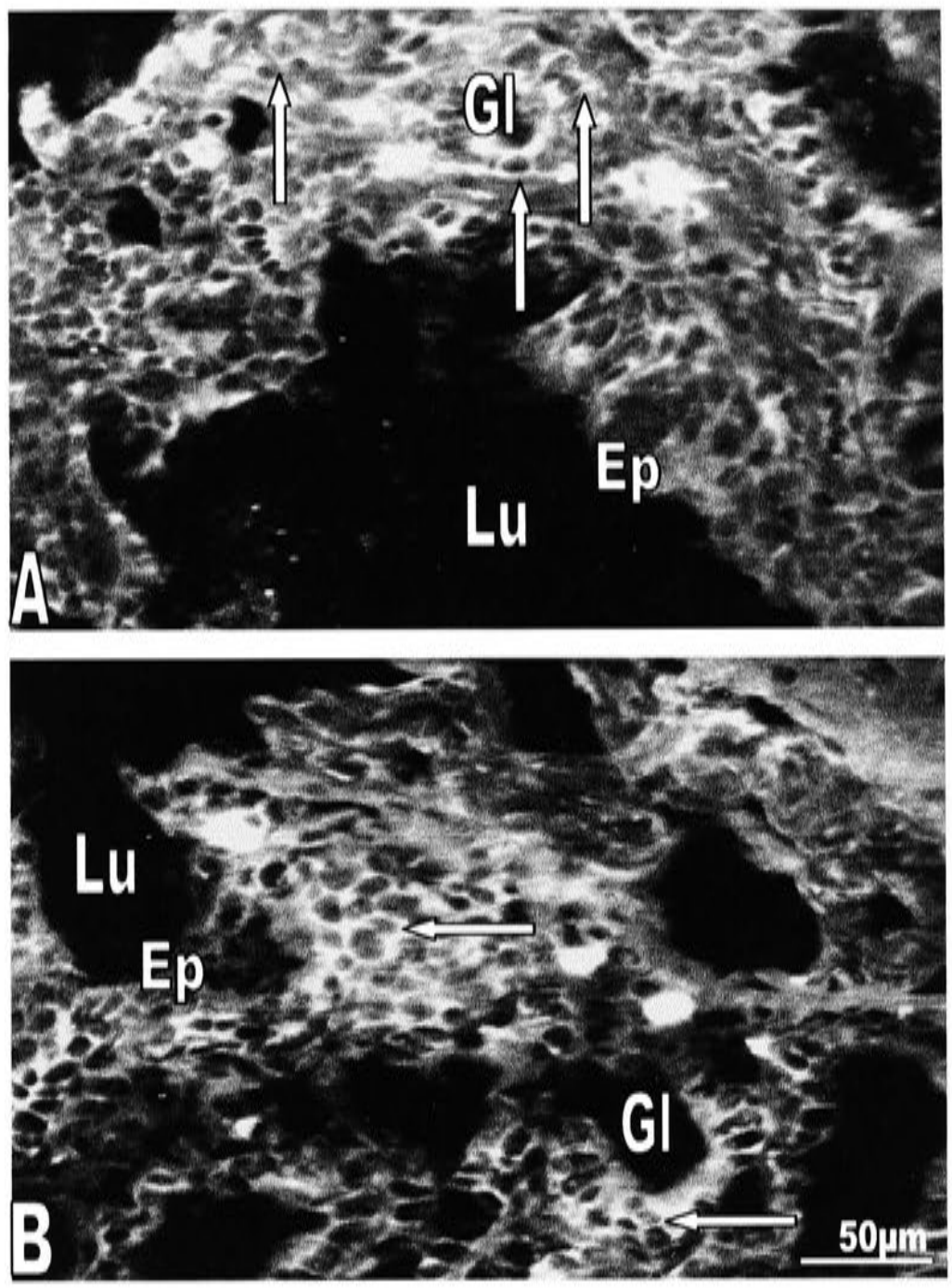


Figure 3-17. Positive control, IgA staining in small intestine. Immunofluorescent images show IgA in small intestine as a positive control for IgA staining. A and B are from individual rabbits. Note: many IgA plasma cells in the mucosal area of the small intestine (arrows) in association with glands (A, arrow) or glandular epithelial cells (B, arrow). Lu: lumen; Ep: luminal epithelium; Gl: gland. All images are at the same magnification.

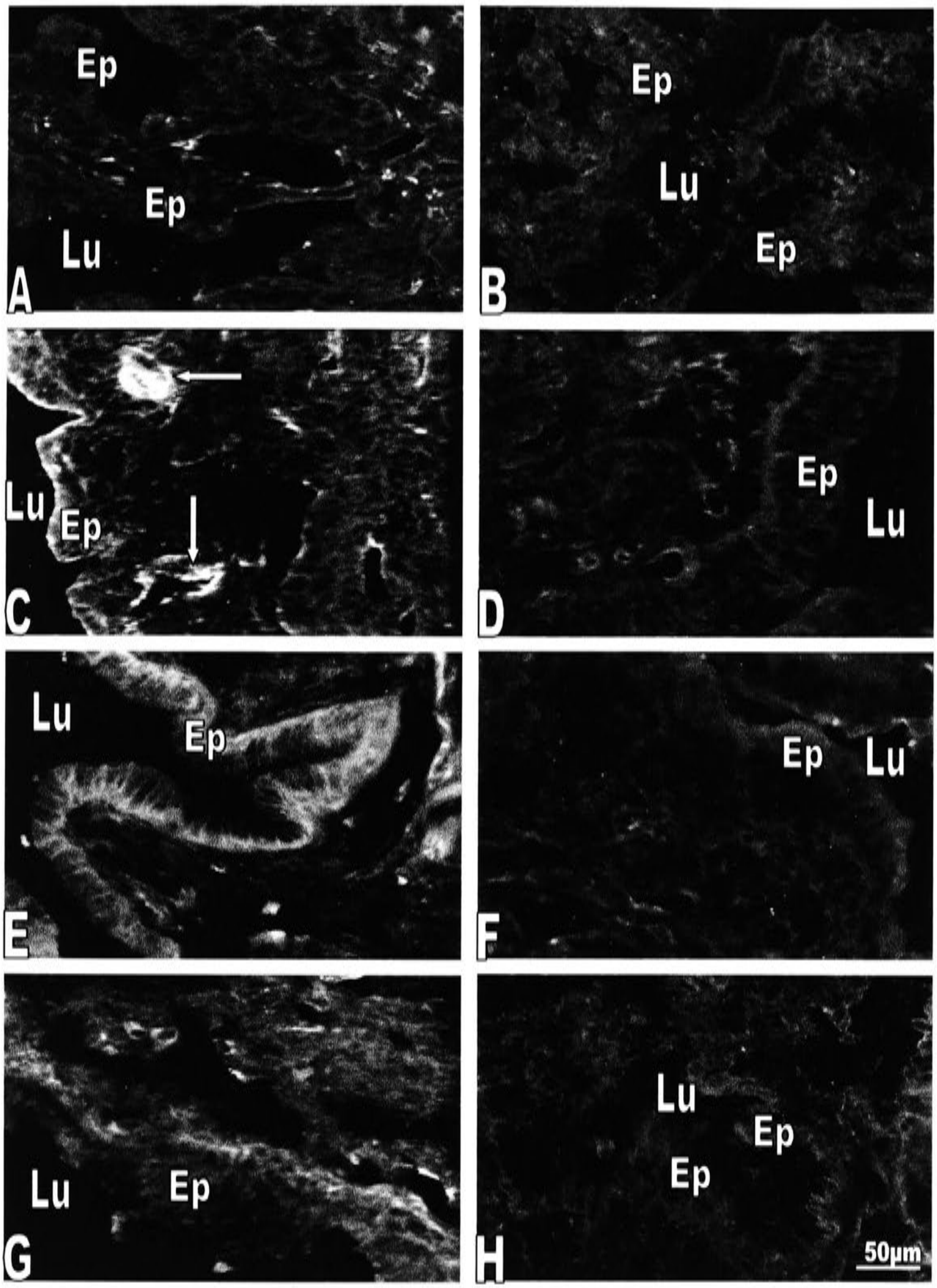


Figure 3-18. IgA staining in the reproductive tract of ovulated rabbits. The four immunofluorescent images on the left show IgA staining in the ovulated reproductive tract and the four images on the right are the MAF-treated negative controls. A and B: oviduct; C and D: uterus; E and F: cervix, and G and H: vagina. Note: IgA staining along the apical edge of the luminal epithelium in the uterus (C), on the epithelial surface of glands (arrows) and along the basal membrane region of the luminal epithelium of the cervix (E). There is a small amount of non-specific background staining in the negative control for the uterus (D). Lu: lumen; Ep: epithelium. All images are at the same magnification.

staining was seen along the basal membrane of the epithelial cells in the cervix (Figure 3-18). The negative controls stained with normal goat serum showed no positive cells or staining on the uterine and cervical epithelia (Figure 3-18).

After induction of ovulation, there was an increase of IgA staining in the deep muscular layer of the oviduct and in the mucosa muscularis of the vagina, compared with that of normal rabbits (Figure 3-19). Because IgA plasma cells did not increase, this observation suggests that IgA transport from circulation into the reproductive tract tissues may increase after induction of ovulation.

3.3.9. IgG containing cells in the reproductive tract

Unlike other mucosal sites where IgA predominates, IgG was the dominant antibody class in the female reproductive tract of rodents (Parr and Parr, 1996). Both serum and local IgG plasma cells in the reproductive tract could contribute to the IgG antibody present in the luminal fluids of the tract (Chapter 1 section 1.4.1). To examine the presence of IgG containing cells and IgG distribution in the reproductive tract, goat anti-rabbit IgG antibody (Chapter 2 Table 2-2) and indirect immunofluorescence (Chapter section 2.4.1) were employed. Normal goat serum (Nordic) was used in negative controls.

3.3.9.1.IgG containing cells in normal reproductive tract

Compared with IgA (section 3.3.8), IgG staining was stronger in all regions of the reproductive tract (Figure 3-20). In addition, IgG positive (plasma) cells were more easily found in most regions of the reproductive tract than IgA positive cells (Figure 3-20 and 16). However, IgG staining in each region of the tract was slightly different.

In the oviduct, strong IgG staining was seen in the lamina propria and sometimes also in the muscular layer but the luminal epithelium was basically IgG negative (Figure 3-20). Because of the strong, widespread IgG staining, the identification of individual IgG positive cells was impossible in this region (Figure 3-20). In the uterus, strong IgG

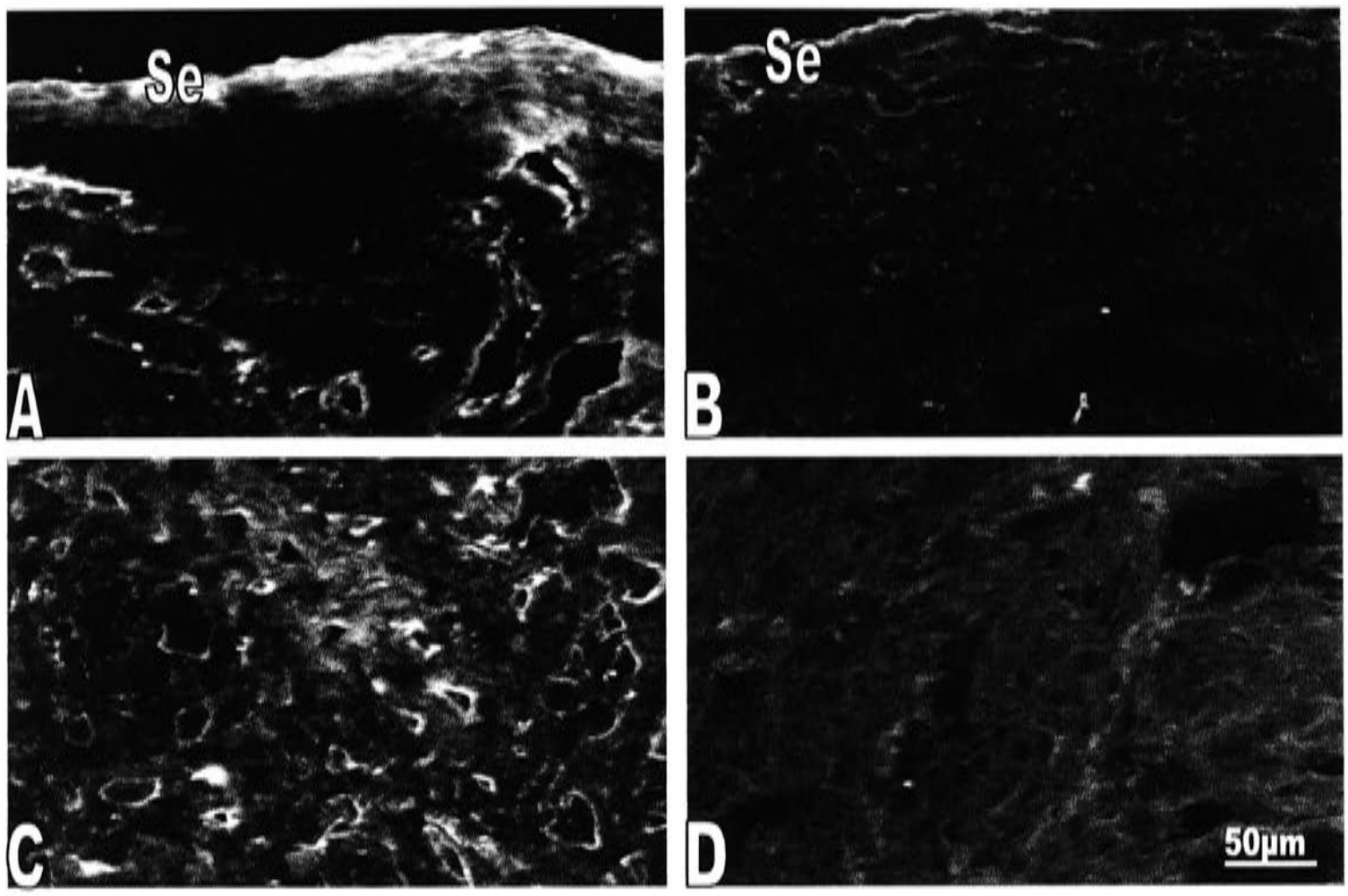


Figure 3-19. IgA staining in the deep muscular layer of the oviduct and in the mucosa muscularis of the vagina after induction of ovulation. Immunofluorescent images show the increase of IgA staining in the deep muscular layer of the oviduct (A) and in the mucosa muscularis of the vagina (C) after induction of ovulation compared with before ovulation (B and D) Se: serosa. All images are at the same magnification.

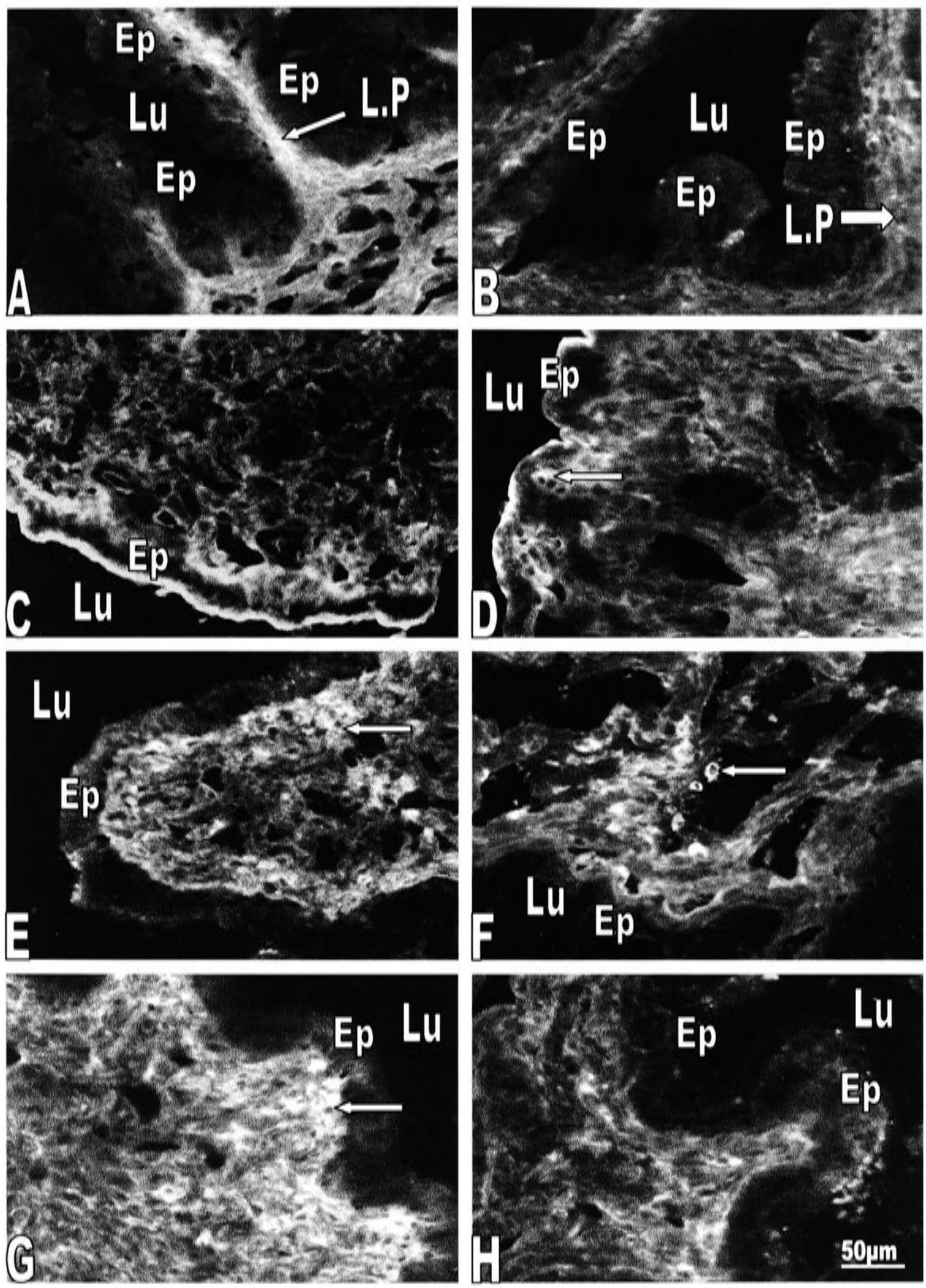


Figure 3-20. IgG staining in the female reproductive tract of normal and ovulated rabbits. The four immunofluorescent images on the left (A, C, E, G) are from normal rabbits and the four on the right (B, D, F, H) are from ovulated rabbits. A and B are oviduct; C and D are uterus; E and F are cervix, and G and H are vagina. Lu: lumen; Ep: luminal epithelium; L.P: lamina propria; arrows: IgG positive cells. All images are at the same magnification.

staining was seen along the surface of the luminal epithelium, but IgG positive cells were not commonly seen in this region. In the cervix and vagina, IgG staining and individual IgG positive (plasma) cells were clearly restricted to the sub-epithelium or stroma rather than the epithelium (Figure 3-20). More IgG positive cells were present in the vagina than the cervix (Figure 3-20) and the epithelia of some glands in the cervix were also IgG positive. When normal goat serum was applied as a negative control, no positive cells or staining were observed and the stained sections were similar in appearance to those shown in Figure 3-16.

3.3.9.2.IgG containing cells in the ovulated reproductive tract

The intensity of IgG staining in all regions of the reproductive tract decreased following induction of ovulation (Figure 3-20). In the oviduct, it was clear that although IgG staining in the lamina propria was much reduced, weak IgG positive cells were frequently present. In the uterus, IgG staining along the surface of the luminal epithelium was still observed though it was weaker than in normal rabbits. In the cervix and vagina, the staining of IgG in the sub-epithelium was reduced compared with that of normal rabbits. However, more IgG positive (plasma) cells were seen in the cervix of ovulated animals compared to non-ovulated animals. This was probably due to the decrease of IgG background staining that made the identification of IgG positive cells easier. But in the vagina, the reduction of IgG staining was also accompanied by a reduction in the number of IgG positive cells (Figure 3-20). When normal goat serum was applied to the sections as a negative control, no staining was observed and the sections appeared similar to those shown in Figure 3-18.

3.3.10. IL-2 mRNA expression in the uterus

IL-2 is an important Th1 cytokine involved in T-cell activation (Roitt, 1997). To examine IL-2 production in the uterus, the level of IL-2 mRNA expression was assessed in frozen sections of the uterus from normal and ovulated rabbits using *in situ* hybridisation with sense and anti-sense IL-2 RNA probes (Chapter 2 section 2.13). As positive controls, frozen sections from popliteal lymph nodes were used. Strong IL-2 mRNA expression was found on sections of popliteal lymph nodes hybridised with the

anti-sense RNA probe; only a few very weak positive signals were seen on comparable sections hybridised with sense RNA probe (Figure 3-21). This demonstrated the specificity of the in situ hybridisation method.

On the uterine sections of normal rabbits (4 rabbits) IL-2 mRNA was not strongly expressed (Figure 3-21) and there was no signal on sections hybridised with sense RNA. IL-2 mRNA expression was substantially increased after induction of ovulation in all 3 rabbits tested. As in normal rabbits, there was no signal on the sections hybridised with the sense probe. The increased IL-2 mRNA expression was mainly concentrated in the deep stroma of one of the three rabbits while in another rabbit IL-2 mRNA was mainly distributed in the areas immediately beneath the epithelium (Figure 3-21). In the third rabbit, IL-2 mRNA expression was found in both deep stroma and sub-epithelium.

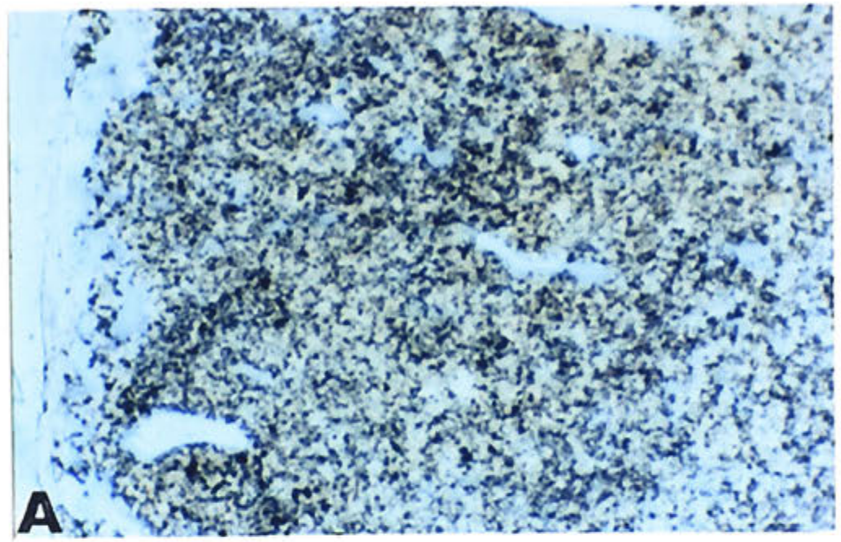
3.3.11. A summary of results:

Histological examination has shown that induction of ovulation has physically expanded the mucosa (lamina propria and epithelium) in most regions of the female rabbit reproductive tract. Immunofluorescent results show the presence of large numbers of CD45+ and CD43+ cells in the mucosal areas of the oviduct, cervix, and vagina but significantly fewer cells in the uterus. Induction of ovulation did not affect the cell density in the mucosal area of each region. CD45+ and CD43+ cells were commonly present in the epithelium: even greater numbers of these cells were present immediately beneath the epithelium of the lower reproductive tract and formed cell clusters. KEN-5+ T cells and MHC-II+ cells were frequently present in the mucosal areas of the reproductive tract but far fewer cells were present in the uterus in normal rabbits. After induction of ovulation the cell numbers of both cell types increased in the uterus.

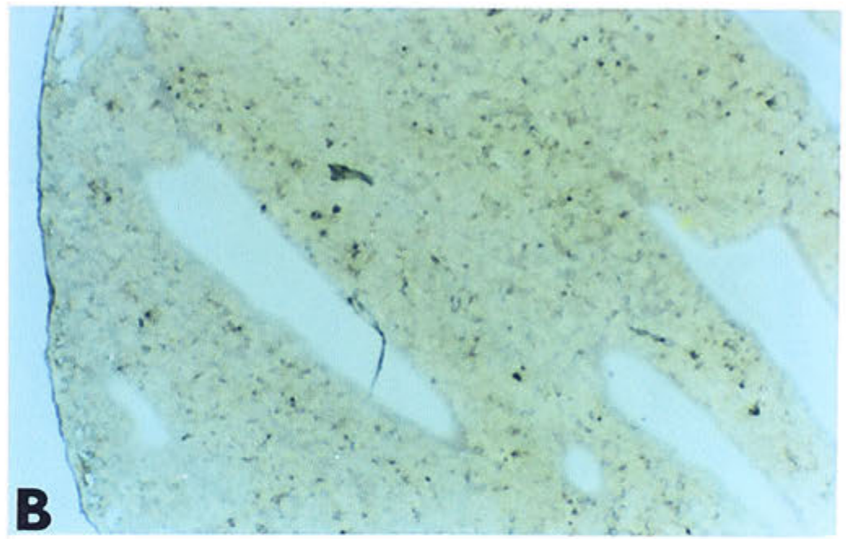
Ig μ-chain and IgA positive B cells were not commonly seen in the reproductive tract and ovulation did not affect this. However, compared with Ig μ-chain and IgA positive B cells, more IgG positive cells were present in the reproductive tract and the

Figure notes are on the other side

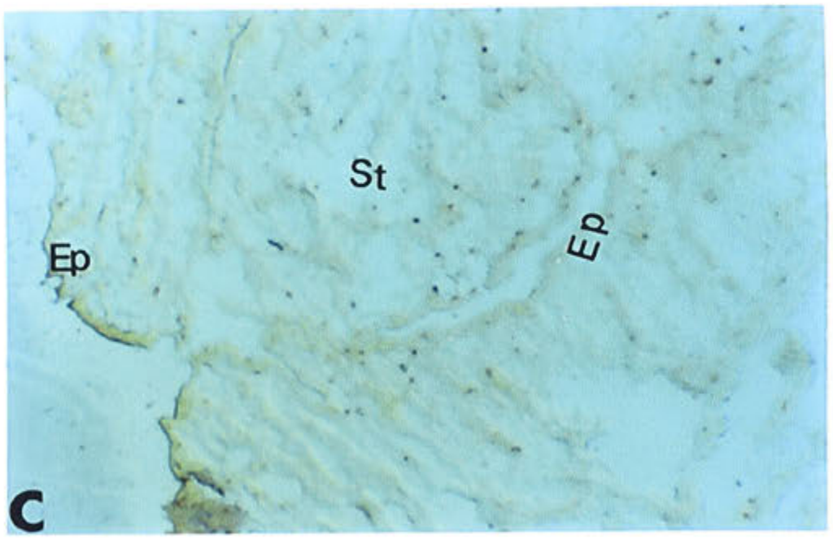
Figure 3- 21. IL-2 mRNA expression in popliteal lymph nodes and the uterus of normal and ovulated rabbits. A represents the lymph node section hybridized with IL-2 anti-sense probe. B represents the lymph node section hybridized with IL-2 sense probe. C represents the normal uterus hybridised with IL-2 anti-sense probe. D represents the ovulated uterus hybridised with IL-2 anti-sense probe. All are 100x magnification.



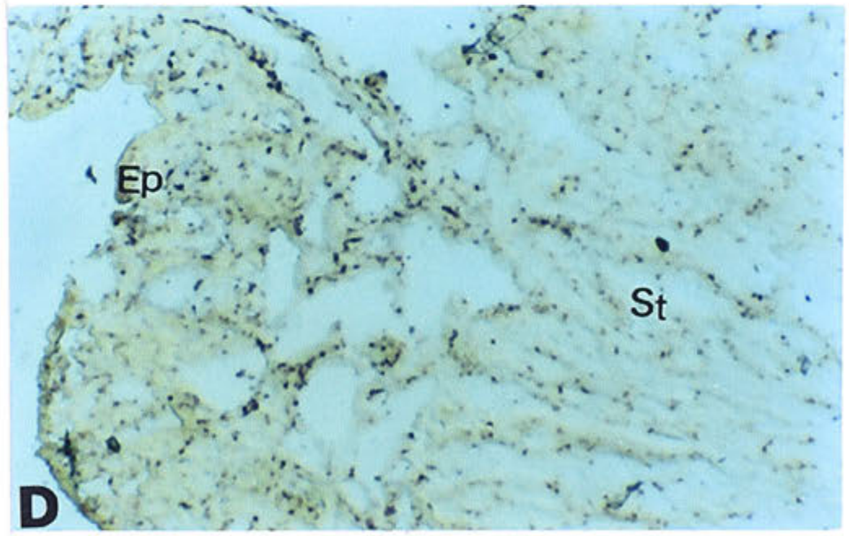
A



B



C



D

vagina contained more cells than other regions. Induction of ovulation decreased IgG staining in the lamina propria of most regions of the tract and IgG positive cell numbers in the vagina but increased IgA staining in the muscular layer of the oviduct and the mucosa muscularis of the vagina. Low level IL-2 mRNA expression was seen in the normal uterus but this dramatically increased after ovulation.

Comparing between the regions, the vagina and cervix contain the greatest number of immune cells, the uterus contains the lowest number of immune cells under oestrogen dominated conditions and the oviduct is intermediate. However, the uterus shows the most sensitive reactions to ovulation with the numbers of KEN-5+ T cells and MHC II+ cells being increased in the endometrium after induction of ovulation and IL-2 expression being strongly up-regulated.

3.4. Discussion

3.4.1. Leukocytes and T cells in the reproductive tract

Understanding the immunology of the female reproductive tract is a first step in rational design of immunocontraceptive vaccines. To achieve fertility control using immunological means in the reproductive tract of the female rabbit, it is necessary that we understand the local immune response in the tract. However, there has, until now, been relatively little interest in rabbit reproductive immunology and, in contrast to the situation in rodents and humans, information on the immunology and immune responses of the female rabbit reproductive tract is very limited. In addition, since the rabbit is an induced ovulator and does not exhibit a regular oestrous cycle like rodents and humans, immune responses in the rabbit reproductive tract could be very different from these species. In this chapter, the basic immune cells in the rabbit reproductive tract have been examined and the available reagents were characterised under the normal condition of oestrogen dominance and after induction of ovulation by hCG injection. Because this study was directed at examining the immunology of the reproductive tract with a view to developing an anti-fertility response, immune cells in the reproductive tract of pregnant rabbits were not examined.

Numerous CD45+ cells were present in the mucosal areas of the vagina, cervix, and oviduct, both before and after ovulation. CD45 is expressed on all leucocytes and is also referred to as the common leucocyte antigen. However, compared with other regions of the reproductive tract, the rabbit uterus contains significantly fewer leukocytes. This contrasts to the finding in human where CD45+ cells were more abundant in the endometrium and fallopian mucosa than in the cervix or vagina (Givan *et al.* 1997) and reflects the variation between species on the distribution of CD45+ cells within the tract. Leucocytes are also abundant in the vagina and cervix of mice (Parr and Parr, 1991) and in the uterus and vagina of rats (Kaushic *et al.* 1998).

CD45 is expressed on B and T lymphocytes, monocytes, granulocytes and thymocytes. To specify more precisely the types of leucocyte present, sections of the reproductive tract were stained with the Mab L11/135 which recognises the CD43 antigen. This antigen is predominantly expressed on T cells in rabbits although Mab L11/135 can also bind weakly to macrophages and monocytes (Wilkinson *et al.* 1992). The staining result showed that CD43+ cells constituted 30-77% of the total CD45+ leucocyte population in rabbits. Because most CD43+ positive cells labelled with L11/135 showed strong fluorescence and were mostly round in shape (Figure 3-7), the majority of the positive cells are probably T cells.

In humans, CD3 (T cell specific marker) positive T cells were shown to be the dominant population (30-60%) of CD45+ leucocytes in the reproductive tract (Givan *et al.* 1997). In addition, T cells are also the predominant lymphocytes in the female reproductive tract of mice (Parr and Parr, 1991; Nandi and Allison, 1993), cows (Cobb and Watson, 1995), and mares (Watson and Thomson, 1996). Thus, it is a common feature for many species, including the rabbit, that T cells are the predominant lymphocytes in the female reproductive tract.

T cell functions such as lysing target cells for CD8+ T cells or producing cytokines for both CD4+ and CD8+ T cells can be associated with their locations in the reproductive tract. In rhesus macaques, T cells isolated from vaginal epithelium (IEL), but not sub-mucosa, could lyse antigen-specific cells (Lohman *et al.* 1995). As these cells were responding to simian immunodeficiency virus antigen, it is possible that IEL

are key effectors for cell and viral immunity. In humans, IEL in proliferative endometrium (before ovulation with oestradiol dominance) consist mainly of T cells, predominantly of the CD8+ subset. It has been suggested that these cells regulate the immune response to implantation (Pace *et al.* 1991). In rabbits, many CD43+ T cells were also located within the epithelium (Figure 3-7) but the functionality of these cells has not been examined.

As well as IEL, in rabbits the leucocytes and CD43+ cells immediately beneath the epithelium (sub-epithelial T cells) of the vagina or cervix could also be important in the immunity of the reproductive tract. There are more of these sub-epithelial T cells than IEL and they are located close to the epithelium (Figure 3-6). In humans, T lymphocytes were found to cluster in a distinct band beneath the epithelium of both cervix and vagina and it was suggested that these cells were not randomly scattered in the tissue but were organised in a distinct pattern (Johansson *et al.* 1999). However, their functional role in local immunity is still not clear. It is possible that these cells have a dynamic relationship with IEL in both rabbits and other species but further studies will be needed to examine this possibility.

Classifying T cell subsets is important for understanding their functions. For example, CD4+ and CD8+ T cells are two major functional T cell sub-populations. CD4+ cells are T helper cells which recognise peptides presented by MHC II on professional APC and provide signals to activate T and B cells. CD8+ cells recognise antigens in association with MHC I molecules and can directly kill these cells, once activated. Both of these broad populations can be further sub-divided into sub-populations including Th1/Th2 and suppressor cells/memory cells. The differences in their distribution in each region of the reproductive tract may reflect differences in local immunity in each region. In B cell deficient mice, CD4+ T cells could still maintain immunity to *Chlamydia trachomatis* infection in the genital tract (Johansson and Lycke, 2001) which suggests that CD4+ cells play a major immune effector role in local immunity in the tract. In the current study, anti-rabbit CD4 monoclonal antibody (Chapter 2, Table 2-2) was employed to label CD4+ T cells in the reproductive tract. However, although this Mab binds strongly to the rabbit T cell line RL-5, it did not bind to any cells in frozen sections. Similar negative results have been obtained with primary

rabbit lymphocytes in culture (P. Kerr, unpublished data). These data suggest that a new Mab to rabbit CD4 may be needed to work on frozen sections.

3.4.2. The effect of ovulation on leucocyte and T cell distribution and activation

In many species including rodents and humans the stage of oestrus has a profound effect upon the distribution and function of lymphocytes in the reproductive tract (Parr and Parr, 1991; Kaushic *et al.* 1998; Givan *et al.* 1997; White *et al.* 1997). For example, in mice, significantly ($P<0.05$) more Thy-1+ (T cells and some NK cells) lymphocytes were present in the vagina at dioestrus than during other stages of oestrus (Parr and Parr, 1991). In humans, T cell numbers in the endometrium were significantly lower in the secretory stage (post-ovulation with high level of progesterone) than in the proliferative stage (Givan *et al.* 1997). In rabbits, the induction of ovulation has physically expanded the lamina propria and epithelia of the reproductive tract (Figures 3-1.1, 3-2.1). However, there were no changes in CD45+ and CD43+ cell density. Therefore, since the tissues have expanded, ovulation must be associated with a substantial increase in the overall numbers of CD45+ and CD43+ cells in these tissues. Interestingly, in the vagina more CD45+ cells were found around the blood vessels in the mucosa muscularis of the ovulated rabbits than in non-ovulated rabbits, indicating that CD45+ cells were probably moving from blood into the vaginal tissues after hCG treatment. Whether this increase in CD45+ cell number was associated with the elevated progesterone level or blood vessel expansion is not clear because no such increase was observed in other regions. In addition to the general increase in T cell numbers, induction of ovulation also increased the numbers of KEN-5+ cells in the rabbit uterine endometrium. It was concluded that this was most likely due to up-regulation of KEN-5 on resident CD43+ T cells or to immigration of activated T cells since some immigration would be necessary to maintain T cell density in the expanded tissues. Otsuki et al (1990) previously showed that T cell numbers increased in the rabbit endometrium by 11 hours after hCG treatment (Otsuki *et al.* 1990). This is consistent with the current findings regarding KEN-5+ cells. However, the increase of T cell numbers in rabbit uterus differs from the findings in humans where the T cell number decreased when the progesterone level increased (Givan *et al.* 1997). These differences between rabbits and rodent or human again reflect a variation between

species in T cell response to the increasing level of progesterone during the oestrous cycle (rodents and humans) or after induction of ovulation (rabbits).

As well as there being an increase in T cells expressing KEN-5, rabbit IL-2 mRNA expression in the uterus increased at the time of ovulation. IL-2 is an early product of activated T cells and a proinflammatory cytokine (Roitt, 1997). Given the increase in KEN-5+ cell number and the increased IL-2 mRNA expression, it is possible that KEN-5+ expression is indicative of T cell activation. These increases could be important for a successful implantation because the normal (oestrogen dominated) uterus contains only a very small number of immune cells including T cells. After ovulation, T cells, probably including immunosuppressive or regulatory T cells increased in number and became activated; this may be important in creating an environment for a successful implantation (Robertson, 2000).

3.4.3. MHC class II expression in the reproductive tract

The ability of cells in the reproductive tract to take up, process and present antigens is essential for induction of a local immune response in the tract (McGhee *et al.* 1994). MHC class II molecules on APC present processed antigen peptides in a MHC-peptide complex to CD4+ T cells followed by T cell receptor recognition and activation of antigen-specific T cells (Chapter 1 section 1.5). On the other hand, antigen presentation in the absence of correct signalling (eg CD40-CD40L interaction) may lead to T cell anergy. Therefore, detection of MHC class II expression in the reproductive tract provides a starting point for determining the numbers of potential APC in the reproductive tract. In rabbits, MHC II+ cells were frequently seen in the mucosa of oviduct, cervix, and vagina under both oestrogen dominated and ovulated conditions but were seen frequently in the uterus only after ovulation (Figure 3-11). MHC II+ cells could include activated T cells, B cells, and APC (Mage, 1998). It was shown in the current study that KEN-5+ cells could be MHC II+ in the normal reproductive tract (Figure 3-13). However, the majority of MHC II+ cells in the vagina and cervix were KEN-5 negative (Figure 3-13).

Uncommitted B cells were rare in the female rabbit reproductive tract based on Ig μ-chain staining and IgA plasma cells were also uncommon (Figures 3-14, 3-16). Although IgG positive cells were frequently seen in the normal vagina they were distinguished by being round-shaped and not located in the luminal epithelium (Figure 3-20g). Since most MHC II+ cells in the vagina or cervix were KEN-5- and given the rarity and location of B cells, most MHC II+ cells in these regions, especially those in or very close to the luminal epithelium, are potential APC. From their irregular cell shape (Figures 3-11e, f, g, h), they are potentially tissue macrophages or professional APCs such as dendritic or Langerhans' cells. In humans, MHC II+ cells were shown to be abundant in the lamina propria of both the cervical and vaginal mucosa. In the epithelium of these tissues, positive cells were found at a mean frequency of 94 cells per mm^2 (Johansson *et al.* 1999). Similarly in rat, numerous MHC II+ cells were present in vaginal sub-epithelial layers and the number was greater than that in the uterus (Kaushic *et al.* 1998). MHC II+ cells were also reported in the epithelia and stroma of the mouse vagina (Parr and Parr, 1991). These data collectively suggest that most species, including rabbits, have an abundant MHC II+ cell presence in vagina or cervix.

In the rat, Wira et al (2000) showed that vaginal macrophages and dendritic cells have the ability to present antigens (ovalbumin) to T cells *in vitro* (Wira *et al.* 2000). Similarly, epithelial or stromal cells in the vagina expressed MHC II and were capable of presenting antigens in the female human reproductive tract (Fahey *et al.* 1999). By extension to the rabbit, APC in vagina and cervix could present antigens introduced into the vagina to T cells in the draining lymph nodes and initiate an immune response (McGhee *et al.* 1994). It remains to be determined whether the effector cells can then migrate back to the vagina and cervix.

3.4.4. The effect of ovulation on MHC II expression

In rabbits, induction of ovulation increased the number of cells in the uterine endometrium which expressed MHC class II (Figures 3-11, 12). It is possible that these positive cells were derived from blood such as migrating macrophages or were activated T lymphocytes expressing MHC-II. But it is also possible that these positive cells resulted from up-regulation of MHC II on local epithelial cells. In the rat, uterine

epithelial and stroma cells could present antigen (ovalbumin) to ovalbumin-specific T cells and stimulate T cell proliferation; this ability was MHC II dependent and could be blocked by MHC II antibody (Wira and Rossoll, 1995b). It is therefore assumed that these cells express MHC II. In humans, freshly isolated uterine epithelial cells were MHC II positive (Wallace et al. 2001). In rabbits, it is possible that the increase of MHC II expression in the uterus reflects an increase of MHC II expression on local epithelial cells. This was supported by the observation that most MHC II+ cells in the uterus were associated with luminal and glandular epithelium (Figures 3-12 c, d). If this is correct, the antigen presentation ability of rabbit uterine epithelial cells could be determined as in the rat study (Wira and Rossoll, 1995b). But there is a potential difference between rabbits and rats. In the rat, the antigen presenting ability of uterine epithelial cells was high at late dioestrus when oestradiol levels are increasing (Wira and Rossoll, 1995b). However, in rabbits, MHC II expression increased in the uterus after ovulation when the progesterone level increases, and so antigen presenting ability is most likely high after ovulation. Whether this indicates that MHC-II expression and antigen presentation on rabbit uterine epithelial cells could be involved in T cell activation, or potentially T cell anergy due to lack of co-stimulation molecules, has not been investigated.

In either situation, the increased MHC II expression on rabbit uterine cells suggests that the uterus is more immunologically active after ovulation and this agrees with the up-regulation of KEN-5+ T cells and increased IL-2 mRNA expression in the uterus after ovulation. All these up-regulations in the uterus driven by the increasing level of progesterone may be an essential step for building up an immune balance between tolerance (to the foetus) and immunity against infection and may be a necessary inflammatory response in preparing for implantation.

Apart from the uterus, no increase of MHC II expression was seen in other reproductive tract regions of rabbits. However, in other species, the ovarian hormone progesterone has been reported to affect MHC II+ expression in the vagina. For instance, in the mouse, more MHC-II+ and F4/80+ (macrophage specific marker) dendritic cells were present in both cervix and vagina at dioestrus than at prooestrus or oestrus (Parr and Parr, 1991). In the rat, MHC II+ cell numbers in vagina were greater at dioestrus than at oestrus (Kaushic et al. 1998). This indicates a clear species difference that may be associated with differing immune capacities at different stages of the

oestrous cycle. It is probable that APC and immune responses would be more important after mating when large amounts of foreign material are deposited in the vagina and there may also be a greater likelihood of trauma and infection. Why these cells are not up-regulated in the rabbit is not at all clear.

3.4.5. IgA and IgG positive cell distribution in the reproductive tract

In the rabbit, uncommitted B cells were uncommon in the normal female reproductive tract as judged by Igμ chain expression. IgA-expressing plasma cells were also uncommon (Figure 3-16) although IgG plasma cells were present in vagina and cervix (Figure 3-20). B cells expressing the Ig μ chain have been described in rabbit uterus (Otsuki *et al.* 1990) and IgG plasma cells have been described in the vagina (Symons and Herbert, 1971). This is consistent with the current result. These findings suggest that local production of IgA is not significant in the rabbit reproductive tract but that local expression of IgG is potentially important in the cervix and vagina.

In contrast, there was a strong staining for both IgA and IgG at both basal and luminal surfaces of the uterine epithelium, indicating that mucosal barriers of IgA and IgG may well be important in the uterus and possibly also in more distal regions of the tract if the antibodies are carried along in reproductive tract secretions. The low numbers of plasma cells, especially in the uterus, indicate that these antibodies are most likely derived from the serum and pass through the epithelial cells by the well-characterised passive transfer or poly-Ig transporter mechanisms (Chapter 1 section 1.4).

There are considerable species differences in the distribution of plasma cells in the reproductive tract. In mice, IgA plasma cells predominated in the uterus at pro-oestrus and oestrous (Rachman et al. 1983) or at all stages of the oestrus cycle (Parr and Parr, 1985). The uterus and oviduct also contained more IgG and IgA plasma cells than did the cervix and vagina (Parr and Parr, 1985). In humans, IgA plasma cells predominated in the fallopian tube (Kutteh et al. 1990). Compared with these species, the rabbit differs in both the numbers and location in the distribution of IgG and IgA positive cells in uterus and oviduct. The plasma cells in the upper reproductive tract of these species can

contribute to local Ig production but this is not the case for rabbits because of the lack of IgG+ plasma cells. But IgG plasma cells found in normal rabbit vagina may contribute to local IgG production in this region. In the rat, IgA plasma cells were absent from the whole female reproductive tract. It was suggested that the IgA present in the tract was mainly derived from serum (Parr and Parr, 1989). Like the rat, the rabbit has few if any IgA positive cells in the reproductive tract. Therefore the IgA in rabbit reproductive tract is more likely to be derived mainly from serum. This would explain why the epithelial surfaces of the rabbit uterus were covered with IgA under normal oestrogen dominated conditions, despite the lack of local IgA plasma cells. In addition, compared with the vagina, far fewer IgG positive cells were present in the upper reproductive tract of the rabbit, but strong IgG staining was present along the surface of the uterine epithelium (Figure 3-20c). This IgG is also probably largely derived from serum, with perhaps some contribution from local plasma cells.

3.4.6. The effect of ovulation on IgA and IgG presence in the reproductive tract

In rabbits, induction of ovulation decreased IgG positive cell numbers in the vagina but did not influence the number of IgA and IgG positive cells in other reproductive tract regions. However, there were very few IgA and IgG positive cells in either uterus or oviduct at any stage. In rodents, oestrous cycles have significant effects on IgG and IgA plasma cell distribution in the uterus. For example, in mice, numerous IgA plasma cells were found in the uterus at pro-oestrus and oestrus but much fewer at other stages of oestrus (Rachman *et al.* 1983). IgA and IgG plasma cells were also reported to increase markedly in mouse uterus between day 1 and days 4-5 of pregnancy (Parr and Parr, 1985). In the rat, oestradiol treatment of the ovariectomised rat increased the infiltration of IgA and IgA plasma cells into the uterus (Wira and Sullivan, 1981), though the uterus is lacking IgA plasma cells during the normal oestrous cycle (Parr and Parr, 1989). This indicates that the rabbit is quite distinct from the common rodent models of reproductive tract immunity, both in terms of total leucocytes and in terms of plasma cells.

An increase of IgA staining in the deep muscular layers of the oviduct and mucosa muscularis of the vagina was observed after induction of ovulation in the rabbit (Figure 3-19). This staining was not associated with a specific cell type, suggesting that IgA transudation from serum to tissue was enhanced by hCG treatment. In contrast to IgA, IgG staining weakened in the lamina propria or stroma of oviduct, cervix and vagina after induction of ovulation (Figure 3-20). However, no such changes were observed in the uterus. In rats, in contrast, IgG and IgA levels in uterine tissues were high at pro-oestrus, IgA remained high but IgG dropped at oestrus, and both IgA and IgG were low at dioestrus (Wira and Sandoe, 1980). Similar results were reported in the mouse (Rachman *et al.* 1983). These comparisons suggest that the reaction of the rabbit uterus to ovulation is different from rodents, which may once more reflect a variation between species or the difference in endocrine status between cyclic oestrus (rodents) and induced ovulators (rabbits).

3.4.7. Epithelial surface IgA and IgG and the specificity of the staining

An interesting observation in the current study is the presence of both IgA and IgG along the epithelial luminal surfaces of the uterus (Figures 3-16c, 3-18c, 3-20c). The staining was mainly located in the apical area of the epithelial cells but sometimes also in basal areas. It is unlikely that this staining was non-specific. Firstly, there was little or no background staining on the negative controls (Figure 3-16), suggesting that the antibody binding is specific. Secondly, on the antibody treated sections, the staining was seen only on the cells or on specific parts of the tissue but not all over the section, suggesting again the specific binding of the antibody. In addition, on sections of the small intestine the antibody reaction was mainly on the plasma cells in lamina propria or around the glands but not on the epithelial luminal surface (Figure 3-17). These data confirm that this antibody specifically reacts with IgA in frozen sections.

It is also highly unlikely that this staining is an edge effect of the antibody conjugate because no such staining was found on the edges of sections from other reproductive tract regions in the same assay, eg. the oviduct and vagina (Figures 3-16a, g and Figures 3-20a, g). In the cervix (Figure 3–18e) in which the epithelial cells are similar to those in the uterus, the staining was clearly seen in the basal area of the epithelial cells

following ovulation but not at the luminal surface. Similarly, IgG staining in the vagina was restricted to the sub-epithelium with no staining in the epithelium or at the luminal epithelial surface (Figure 3-20g). These data collectively confirm the staining is specific and not an edge effect.

The surface IgA and IgG are either within the epithelial cells or very tightly attached to the cell surface because these molecules were not removed by fixation and repeated washing during section preparation and immunofluorescent staining (Chapter 2, section 2.2.3.3 and 2.4.1). Such staining has not been documented in other species and it appears that this is a special characteristic of rabbits. In mice, during oestrus, the lumen of reproductive tract was full of fluid (Parr and Parr, 1998) but this was not observed in rabbits either before or after ovulation. This suggests the secretion mechanism of the uterine epithelial cells of rabbits is different from mice. More investigation is needed to address these issues.

3.4.8. Conclusion

It is clear in rabbits that each region of the female reproductive tract has its unique pattern of immune cell distribution, a pattern that may well be linked to its immune function. In the female reproductive system, the vagina and cervix are anatomically at a position frequently exposed to a variety of antigens (including bacteria and viruses) either from faecal or environmental contamination or at mating where sperm are also antigenically foreign. In the rabbit, the vagina and cervix contain more leukocytes, T cells, potential APC and plasma cells than other regions of the reproductive tract and are more likely to have the capacity to mount an immune response against infection. Rats have a similar immune cell distribution to rabbits (Kaushic *et al.* 1998) and in humans, T cells and APC are also abundant in the vagina (Givan *et al.* 1997). The presence of these cells in the lower reproductive tract of several species is probably an important feature of the defence against external infection.

The uterus is where implantation and fetal development occur. Protecting the "half-self" fetus from immunological rejection by immune suppression and regulation

(Robertson, 2000) and the prevention of infection by active immune responses are both important. Before ovulation when oestrogen is dominant, the rabbit uterus contains small numbers of immune cells so it may be not be immunologically active. However, the uterus is the only region in which immunological changes occur following ovulation. Following hCG treatment, there was an increase of KEN-5+ and MHC II+ cells and IL-2 mRNA expression in the uterus. This could be important for setting up defensive immunity and also contribute to immune regulation for implantation. However, in humans and rodents, other regions as well as the uterus were also sensitive to ovulation (Parr and Parr, 1991; Kaushic *et al.*1998; Givan *et al.* 1997).

The oviduct is the site where fertilisation occurs and the embryos undergo early development. In rabbits, it contains medium levels of immune cells, higher than the uterus but lower than the vagina or cervix. The oviduct is dominated by T cells with less frequent MHC II positive cells and plasma cells. These T cells may be more involved in immuno-suppression or some immune regulatory function that is involved in protecting the conceptus rather than in mounting an immune response against potential infections. In contrast, in humans, the oviduct contains more CD45+ cells (Givan *et al.* 1997) and IgA plasma cells (Kutteh et al. 1990) than the vagina; these are more likely to be involved in local immune responses.

CHAPTER 4: IMMUNE RESPONSE IN THE FEMALE RABBIT REPRODUCTIVE TRACT TO A MODEL ANTIGEN INFLUENZA HA DELIVERED BY A RECOMBINANT MYXOMA VIRUS

4.1 Introduction

Myxoma virus has been chosen as a potential vector to deliver immunocontraceptive vaccines for rabbit population control (Tyndale-Biscoe, 1994; Holland and Jackson, 1994). It has previously been shown in rabbits that both IgG and IgA antibody in the reproductive tract fluids are contributors to blocking fertilisation (Menge and Lieberman, 1974). Therefore, induction of a satisfactory antibody response including both IgG and IgA antibody classes in the reproductive tract fluid by the recombinant myxoma virus may well be crucial for the success of viral-vectored immunocontraception acting in the reproductive tract.

However, it has not proved easy to induce a satisfactory antibody response in the female reproductive tract with conventional immunisation regimes (Menge *et al.* 1993; Gallichan and Rosenthal, 1995; Russell and Mestecky, 2002). Many researchers are exploring new ways to enhance the mucosal immune response in the female reproductive tract such as using viral-like particles or synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs (Gallichan et al. 2001; Li, et al. 2001; Dumais et al. 2002; Russell and Mestecky, 2002). Using a viral vector to deliver the antigen is one of such methods (Michalek *et al.* 1994; Gallichan and Rosenthal, 1995; Rosenthal *et al.* 1996; Morrow *et al.* 1996). It is believed that the replication of the

recombinant virus and its ability to penetrate through the mucosal surfaces are essential for stimulating a good mucosal antibody response (Rosenthal *et al.* 1996; Parr and Parr, 1994).

Like many other poxviruses, myxoma virus has been investigated as an antigen delivery vector for vaccines acting through a systemic immune response (Kerr and Jackson, 1995; Bertagnoli *et al.* 1996). Female rabbits infected intradermally with a recombinant myxoma virus expressing influenza haemagglutinin (MV-HA) had a high and sustained IgG response to HA in blood plasma and a detectable level of IgG antibody in vaginal washings (Kerr and Jackson, 1995). However, whether the recombinant myxoma virus could induce an effective immune response with high levels of antibody in the female rabbit reproductive tract has not been determined. This is important in determining whether a strategy for immunocontraception that envisages high levels of antibody in the reproductive tract is practical. In the mouse, recombinant adenovirus is able to induce an effective mucosal immune response in the female reproductive tract (Gallichan *et al.* 1993; Gallichan and Rosenthal, 1995; Xiang and Ertl, 1999). It is necessary to investigate whether a recombinant myxoma virus could similarly deliver an antigen and induce a mucosal immune response in the female rabbit reproductive tract.

IgG antibody in serum can passively transfer into the reproductive tract (Chapter 1 section 1.4.1). Polymeric IgA and IgM produced locally by plasma cells in the lamina propria can be transferred across the epithelium into the reproductive tract lumen through pIgR on epithelial cells (Chapter 1 section 1.4.2). Therefore, how best to present antigen and achieve a maximal IgG antibody transfer into the reproductive tract lumen and have plasma cells homing back to the reproductive tract are major objectives for inducing an effective antibody response in the tract.

The female reproductive tract is considered to be a part of the common mucosal immune system. Studies in both human and mouse have demonstrated that immunisation at distal mucosal surfaces such as the nasal mucosa can be effective in inducing mucosal immune responses in the female reproductive tract (Ogra and Ogra, 1973; Gallichan and Rosenthal, 1995; De Haan *et al.* 1995; Johansson *et al.* 1998; Xiang and Ertl, 1999; Gallichan et al. 2001; Russell, 2002). In mice, for example,

intranasal immunisation with recombinant adenovirus expressing HSV-1 glycoprotein B induced specific and protective IgA and IgG responses in serum and vaginal secretions (Gallichan *et al.* 1993; Gallichan and Rosenthal, 1995). Antigen specific antibody-secreting cells were also found to home back to the reproductive tract after intra-nasal immunization (Gallichan and Rosenthal, 1998). Similar results were obtained in mice with a recombinant adenovirus expressing rabies virus glycoprotein (Xiang *et al.* 1999). In the rabbit, intranasal immunisation with HIV-1 C4/V3 peptide and a subsequent intranasal booster showed better antibody responses in vaginal secretions than intra-vaginal administration and boosting (Zinckgraf *et al.* 1999).

Intra-vaginal immunisation is an effective way of inducing local mucosal immune responses in the female genital tract of mice (Johansson *et al.* 1998, Kato *et al.* 2000), rats (Menge *et al.* 1993), macaques (Lehner *et al.* 1992) and humans (Kozlowski *et al.* 1999; Johansson *et al.* 2001). In addition, neutralising IgA and IgG antibody responses to HIV-1 envelope protein was elicited in mouse vaginal secretions after intra-vaginal immunization with a DNA vaccine (Wang *et al.* 1997). In rabbits, a detectable specific IgA response was induced to horseradish peroxidase in the reproductive tract secretions following intra-vaginal immunization and boosting (McAnulty and Morton, 1978). Results from Chapter 3 (sections 3.3.6 and 3.4) showed that many potential APC (MHC class II positive cells) were present in the lower reproductive tract of female rabbits, suggesting that this part of the tract may have the ability to present antigens to the T cells in local draining lymph nodes and then stimulate a local immune response (McGhee *et al.* 1994).

In rats and mice, IgA antibody titers in reproductive tract secretions were significantly elevated in ovariectomised animals treated with oestradiol (Wira and Sandone, 1987; Wang *et al.* 1996). IgA antibody titers in murine vaginal washings were also significantly higher during oestrus than dioestrus or proestrus but IgG antibody was significantly higher in dioestrus than oestrus (Gallichan and Rosenthal, 1996). These results illustrate the close relationship between endocrine status and the immune response. Unlike these rodents, which exhibit regular oestrous cycles and ovulation, ovulation in rabbits is induced by mating, with oestrogen being the dominant ovarian hormone before mating (Hammond and Marshall, 1925). Although changes in immune cell (including IgG plasma cell) distribution and Ig presence in reproductive tract tissues

were observed after induction of ovulation (Chapter 3 section 3.3.8 and 3.3.9) it was unclear how this would affect the immune response and antibody production in the reproductive tract.

An alternative approach to achieve immunocontraception is to use ovarian antigens, which are accessible to systemic antibodies (Chapter 1 section 1.6.3). If an ovarian antigen were to be vectored by the recombinant myxoma virus for immunocontraception strong antibody responses in serum and ovarian follicular fluids would be essential (Chapter 1 section 1.6.3).

To investigate these issues, a model antigen delivery system of recombinant myxoma virus expressing influenza virus HA was used to infect female rabbits by different routes. Immune responses to HA or myxoma virus were monitored in serum, reproductive tract fluids and ovarian follicular fluids.

4.2 Experimental design

Experiment 1: Eight female rabbits were intradermally inoculated with 10^6 pfu of MV-HA (Chapter 2 section 2.2.2.1) to determine whether a high-level antibody response could be induced in the female rabbit reproductive tract with a natural infection of MV-HA. Four rabbits were killed at 15 days and four at 30 days post-infection, the peak period of specific antibody production (Kerr and Jackson, 1995). Another four female rabbits injected with PBS instead of the recombinant virus served as controls and were killed at 15 days.

Ovulation and its associated hormonal changes influence antibody entry into the female reproductive tract (Chapter 1 section 1.5.3; Chapter 3 section 3.3.8.2, 3.3.9.2). To examine the effect of ovulation on antibody titers, another eight female rabbits were similarly infected with 10^6 pfu of MV-HA and ovulation was induced by intravenous administration of hCG (Chapter 2 section 2.2.2.2) 12 hours before they were killed either at day 15 or day 30 post infection. Another four non-infected rabbits treated with hCG were killed as controls. When the rabbits were killed blood samples were collected for preparation of serum. Luminal fluids were collected from oviduct, uterus and

vagina by both flushing (4 rabbits of each group) and capillary micro-puncture (two rabbits of each group, Chapter 2 section 2.2.3.1). For collection of capillary micro-puncture samples, rabbits were anaesthetised with pentobarbitone; after sampling, they were killed with an overdose of pentobarbitone. Micro-puncture samples were collected by Dr Russell Jones. Tissue samples from oviduct, uterus, cervix and vagina were collected for preparation of frozen and paraffin-embedded sections (Chapter 2 section 2.2.3.2) to examine the cellular response and histological changes.

Experiment 2: To test the ability of immunisation at distal and local mucosal sites to induce an effective mucosal immune response in the female reproductive tract, two groups of 8 female rabbits were used. One group was immunised intra-nasally and another intra-vaginally with 10^6 pfu of MV-HA (Chapter 2 section 2.2.2.1). Blood, nasal and vaginal washings were taken from each rabbit before immunisation. Four rabbits from each group were killed at days 15 and 30 post-infection and blood and reproductive tract flushing samples were collected as described above. In addition, nasal washings (Chapter 2 section 2.2.3.1) were collected for antibody measurement and tissues from each region of the reproductive tract were collected and prepared for frozen and paraffin-embedded sections.

Experiment 3: In order to investigate IgA and IgG titers in reproductive tract fluids over a longer time frame after MV-HA infection or after boosting with HA antigen, four rabbits were immunised with 10^6 pfu MV-HA, two intra-nasally and two intra-vaginally. At day 70 post-infection, these animals were boosted intra-vaginally with influenza virus (Chapter 2 section 2.2.2.1). Blood, nasal and vaginal washings (Chapter 2 section 2.2.3.1) were taken at days 3, 15, 30, 45, 70 after immunization and at days 3, 6, 10 after boosting. These animals were killed on day 15 post-boost and blood samples and reproductive tract flushings were collected.

Experiment 4: Four female rabbits were intradermally immunized with 10^6 pfu MV-HA to compare antibody levels in serum and ovarian follicular fluids. Blood samples were collected before immunization for preparing pre-immune serum and at 15 days after immunisation for antibody assay. For collecting ovarian follicular fluids the animals were injected with PMSG (72 hours before sampling) and hCG (12 hours before sampling) to induce super-ovulation (Chapter 2 section 2.2.2.2). Blood samples

and ovarian follicular fluids were collected for antibody measurement (Chapter 2 section 2.2.3.1).

4.3 Results

4.3.1 Antibody response to HA following intradermal immunisation with MV-HA

HA specific antibody titers of IgG, IgA, and IgM classes in reproductive tract fluids (including flushing and micropuncture samples) and serum were measured using ELISA (Chapter 2 section 2.3.1). High titers of HA specific IgG were detected in serum at both 15 and 30 days post-infection (Figure 4-1). HA specific IgG was also detectable in reproductive tract fluids but the titers were 100-1000 fold lower than those in serum (Figure 4-1). No significant change of IgG antibody titers was detected between day 15 and day 30 in either serum or reproductive tract samples. The titers in samples obtained from different regions of the tract were comparable. No HA specific IgG was detected in serum and reproductive tract fluids from uninfected controls.

HA IgG titers in the micropuncture samples were not different from those in flushing samples in any region (Figure 4-1). The micropuncture samples were collected from free-flowing fluids in the lumen of the reproductive tract (volume of 100-150μl) whereas flushing samples were collected by flushing the lumen with 1ml PBS. Therefore, compared with micropuncture samples the flushing samples were diluted about ten times. However, similar HA IgG titers were obtained in the samples collected by the two different methods, suggesting that flushing samples might contain mucus flushed from the mucosal layer and which contained higher levels of specific IgG than the free fluids (micropuncture samples). This result also suggests that specific IgG titers in flushing samples can be used to represent antibody titers in the reproductive tract free flow fluids.

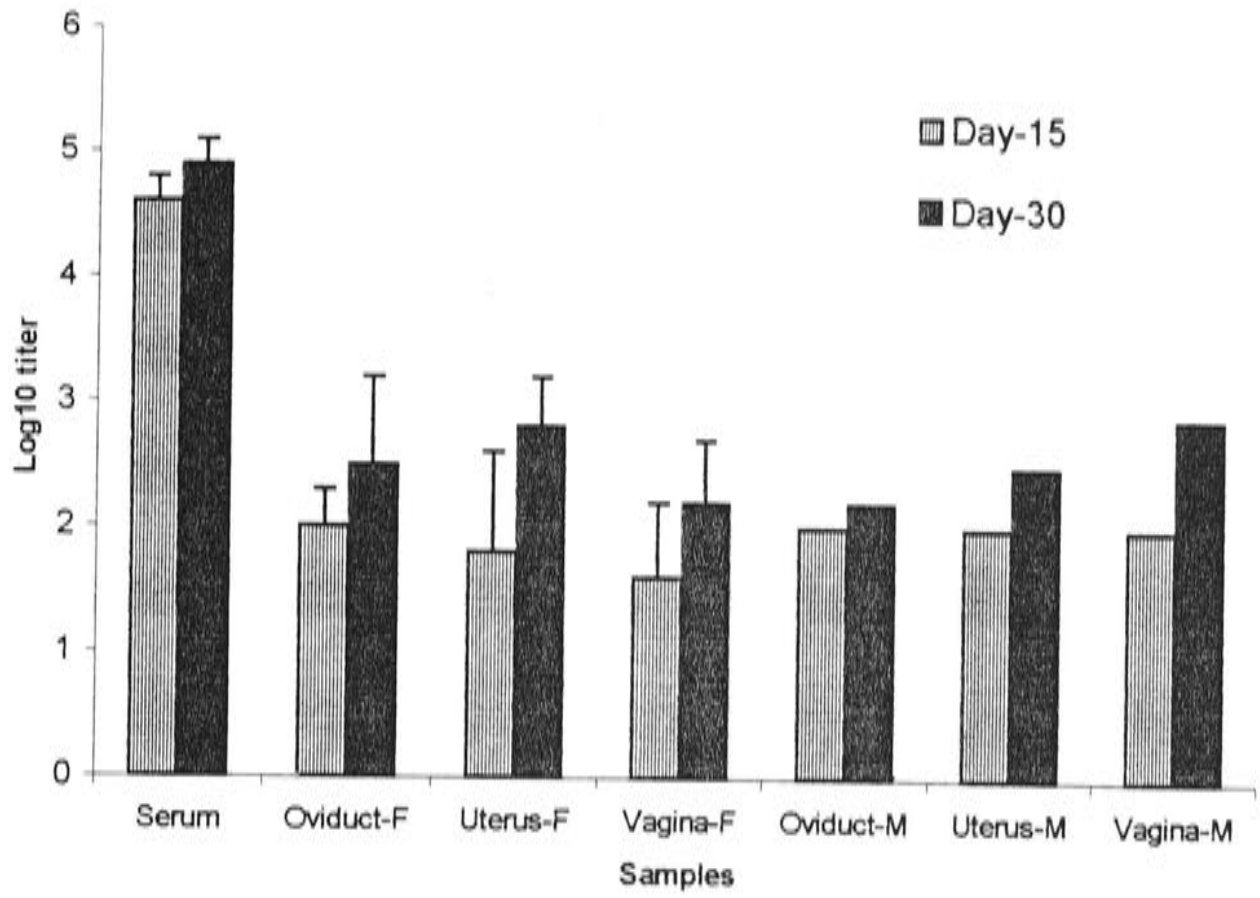


Figure 4- 1. HA specific IgG titers in serum and reproductive tract fluids after intradermal infection with MV-HA

Rabbits were immunised with 10^6 pfu MV-HA. Specific IgG titer to HA was determined by ELISA in serum and reproductive tract fluids collected from oviduct, uterus and vagina by both flushing and micro-puncture at either 15 or 30 days post-infection. The titers were transformed into logarithms and expressed as mean Log_{10} titers for four (flushing samples) or two (micropuncture samples) rabbits. F: flushing samples. M: micropuncture samples. The error bars represent standard deviation.

HA specific IgM was always above 1:100 in serum but was only detected in one oviduct flushing sample at a titer of 1:5. HA IgM was also detected in two micropuncture samples, an oviduct sample from one rabbit and a vaginal sample from a second at the same titer of 1:5. There was no significant difference between the average titer of HA IgM in serum at day 15 (Mean ± S.D, 2.7 ± 0.4) and day 30 (Mean ± S.D, 2.2 ± 0.3) post-infection (P=0.11). HA specific IgM was not detected in serum or reproductive tract fluids from uninfected controls.

HA specific IgA was not detectable in serum or reproductive tract fluids from either infected or control rabbits.

4.3.2 The effect of ovulation on HA antibody titers

HA antibody titers were also measured in serum and reproductive tract fluids in rabbits infected with MV-HA and treated with hCG 12 hours before sampling. HA specific IgG titers in reproductive tract fluids were still much lower than those in serum and were not different from un-ovulated animals at either time point (Figure 4-2). There were no significant differences in HA specific IgG titers between samples collected

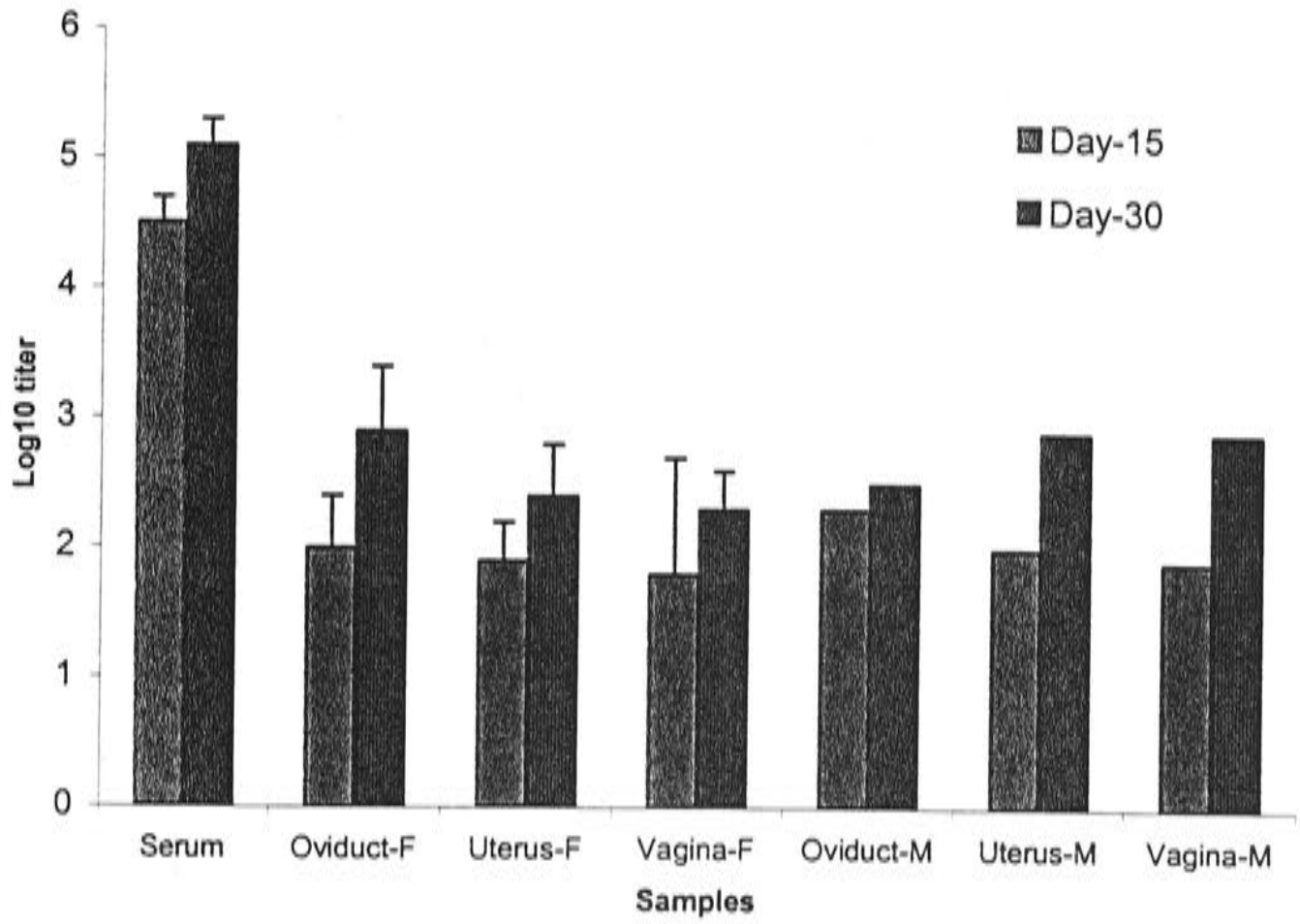


Figure 4- 2. HA specific IgG in serum and reproductive tract fluids of rabbits infected with MV-HA and treated with hCG

Rabbits were intradermally immunised with 10^6 pfu MV-HA. Ovulation was induced by hCG injection 12 hours prior to sampling. Specific IgG titer to HA was determined by ELISA in serum and reproductive tract fluids collected from oviduct, uterus and vagina by both flushing (mean of four rabbits) and micro-puncture (mean of two rabbits) at 15 and 30 days post-infection. The antibody titers were transformed into logarithms and expressed as mean Log_{10} titers for four (flushing samples) or two (micropuncture samples) rabbits. F: flushing samples. M: micropuncture samples.

from different regions of the reproductive tract. However, HA specific IgG titers in serum and oviductal flushings were higher at 30 days than at 15 days ($P<0.05$ for both samples). Similar HA specific IgG titers were obtained in micropuncture samples and in flushing samples (Figure 4-2). No HA IgG was detected in serum or reproductive tract fluids from uninfected animals after induction of ovulation.

HA IgM titers in serum ranged from 1:100 to 1:1600 (Mean ± S.D, 2.7 ± 0.5 and 2.2 ± 0.2 for day 15 and 30 post-infection) whereas HA IgM was only detected in oviductal, uterine and vaginal flushings from a single rabbit at titers of 1:10, 1:10, and 1:40 respectively. These values are similar to those obtained prior to ovulation. No HA IgM was detected in micropuncture samples or serum or reproductive tract flushings from uninfected controls after induction of ovulation.

HA specific IgA was not detectable in serum or reproductive tract samples (flushing or micropuncture) from infected or uninfected rabbits after induction of ovulation.

4.3.3 Antibody response to HA following intranasal infection with MV-HA

Intradermal immunisation did not induce a high level of HA specific IgG in reproductive tract fluids. It produced a poor IgM response and completely failed to stimulate an IgA response in either serum or reproductive tract fluids. In an attempt to provoke an IgA response in the reproductive tract, the rabbits were infected through the intranasal route, an effective inductive site of the common mucosal immune system. As ovulation had not influenced antibody titers in the previous experiment, hCG treatment was not used in these trials.

Between 10-20 days after intranasal inoculation, serous ocular discharges and red conjunctivae were observed in 7/8 of the infected rabbits. This was probably due to local virus replication.

Similarly to intradermal immunisation, intranasal immunisation induced high titers of HA specific IgG in serum and much lower titers in reproductive tract flushings at

both 15 and 30 days post infection (Figure 4-3). No differences were found between samples collected from different regions of the reproductive tract. Compared with intra-dermal immunisation (Figure 4-1), the intra-nasal route elicited higher serum IgG titers at both 15 days ($P<0.01$) and 30 days ($P<0.01$) post-infection. The titers obtained in reproductive tract flushings were comparable to those generated by intra-dermal inoculation, except for titers in oviductal flushings which were higher than in the intra-dermal group at 30 days post-infection ($P<0.05$). HA specific IgG was detectable in nasal washings at similar titers to those detected in reproductive tract flushings (Figure 4-3). No HA specific IgG was detected in serum, nasal and vaginal washings collected before immunisation.

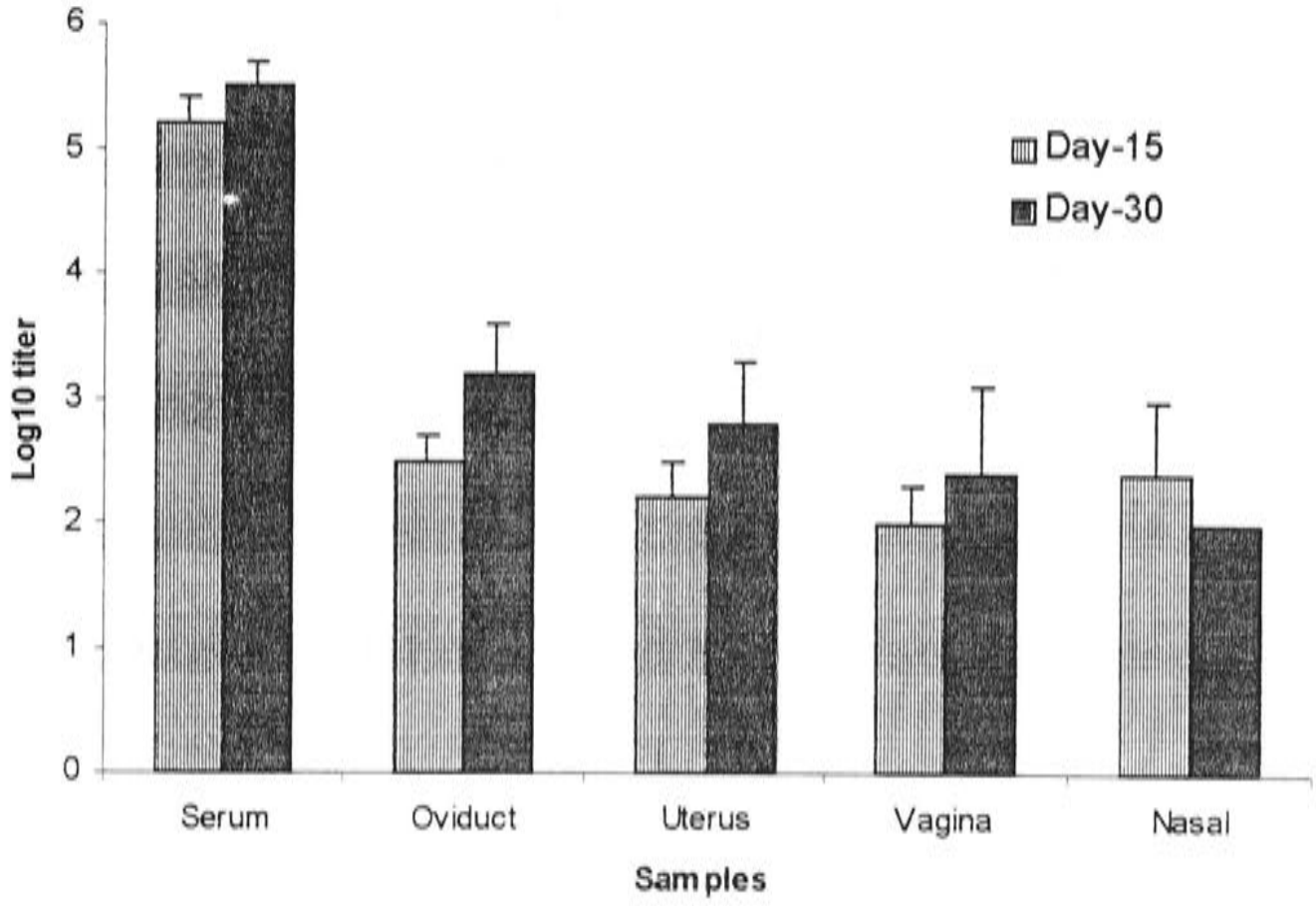


Figure 4- 3. HA specific IgG titers in serum, reproductive tract flushings and nasal washings after intranasal infection with MV-HA

Rabbits were intra-nasally immunised with 10^6 pfu MV-HA. IgG titers to HA were measured by ELISA in serum, reproductive tract flushings (from oviduct, uterus and vagina) and nasal washings at 15 and 30 days post-infection. The antibody titers were transformed into logarithms and expressed as mean Log_{10} titers; n = four rabbits.

The HA specific IgM titer was always above 1:50 in serum (Mean ± S.D, 3.1 ± 0.2 for day 15 group; 1.9 ± 0.2 for day 30 group) but was detected in only one uterine flushing and one nasal washing at a titer of 1:5. The mean titer in serum of rabbits at 30 days post-infection was lower (P=0.001) than that at 15 days post-infection. No specific IgM was detectable in pre-immune serum, nasal washings or vaginal washings.

HA specific IgA was detected in oviductal samples from 4 rabbits and one uterine sample at a titer of 1:3. One nasal washing also showed a HA specific IgA titer of 1:6. No HA specific IgA was detected in any other samples either before or after infection.

4.3.4 Antibody response to HA following intra-vaginal infection with MV-HA

Local immunisation in the female reproductive tract is thought to be capable of inducing antigen presentation in the draining lymph nodes of the reproductive tract (McGhee *et al.* 1994) resulting in B cell homing and local IgA antibody production (McGhee *et al.* 1999, Chapter 1 section 1.5.2). The results from Chapter 3 (section 3.3.5) showed that many MHC II+ cells that are potential antigen-presenting cells were present in the vagina. To examine antigen presentation and local antibody response in the rabbit reproductive tract, a group of rabbits was immunised intra-vaginally with MV-HA.

4.3.4.1 Replication of recombinant virus in the reproductive tract

For intra-vaginal immunisation to be effective, the virus must replicate and produce HA or be transported to and replicate within the cells of the draining lymph node. After immunisation, red and swollen vaginas were observed in 6/8 infected rabbits between 10 and 15 days post-infection. This indicated that the virus might be replicating locally. To confirm this, frozen sections of the reproductive tract tissues were collected at day 15 and day 30 and examined by immunofluorescence (Chapter 2 section 2.4.1) for myxoma virus antigen. Viral positive cells were found in the epithelium and sub-epithelium of the vagina and cervix (Figure 4-4) from rabbits at both infection time points. However, viral positive cells were not found in the oviduct although a few

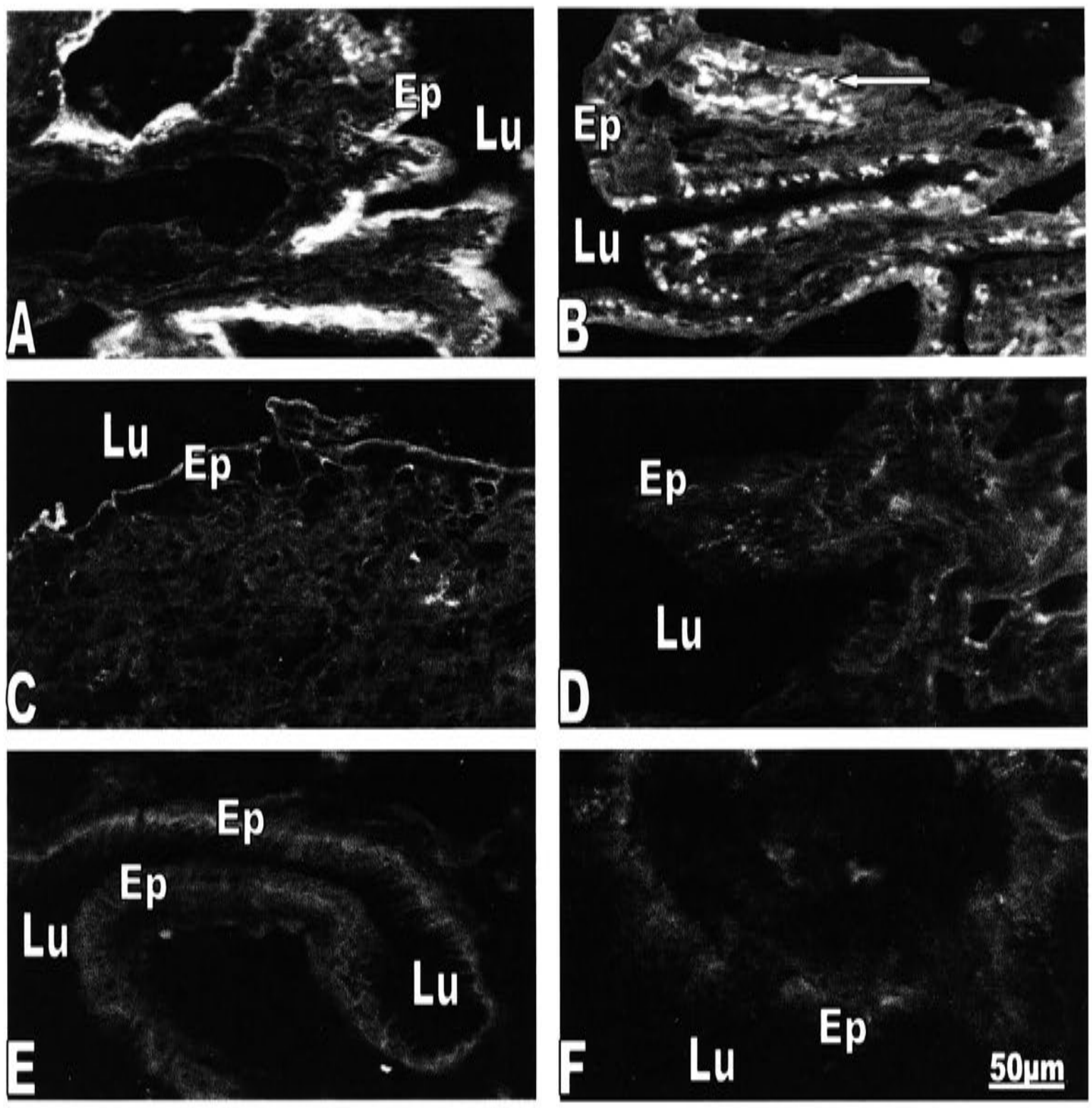


Figure 4- 4. MV positive cells in the reproductive tract of rabbits intra-vaginally immunised with MV-HA. The immunofluorescent images show the presence of MV positive cells in the reproductive tract. A represents the vagina from rabbits 15 days after intra-vaginal immunisation with MV-HA. B represents the cervix from the same group. MV positive cells were also found in the glandular epithelium in the stroma of the cervix (B, arrow). C and D represent the uterus and oviduct from the same infected group. E represents the vagina from intra-nasally immunised rabbits at the same infection time point and shows no MV positive cells were present. F is vagina from an intra-vaginally infected rabbit treated with MAF. Lu: lumen; Ep: luminal epithelium. All images are at same magnification.

positive cells were seen in the uterus of the same infected rabbits (Figure 4-4). As negative controls, frozen sections of reproductive tract tissues from the intranasal and uninfected groups were also examined for the viral antigen. No MV positive cells were found in those sections (Figure 4-4) or in sections treated with MAF, the negative control for the staining, although there is some weak background staining (Figure 4-4).

In addition to MV antigen, HA antigen was also detected in the vagina from the intra-vaginally immunised rabbits using rat anti-HA antibody (Chapter 2 Table 2-2). As predicted, HA positive cells were found in the luminal epithelium of the vagina from these rabbits (Figure 4-5), suggesting that HA was expressed within the virally infected cells. No examination of the draining lymph node was made.

To further identify the viral positive cells, frozen sections of the vagina and cervix from the intra-vaginally immunised group were double labelled with anti-MV and anti-MHC class II monoclonal antibodies using double immunofluorescence (Chapter 2 section 2.4.3). Almost all MV positive cells in these two regions were also MHC class II positive (Figure 4-6) suggesting that recombinant MV has infected MHC class II positive cells in the vagina and cervix and that these cells might then present viral antigens to T cells in the draining lymph nodes of the reproductive tract and initiate the immune response (McGhee *et al.* 1994).

4.3.4.2 HA antibodies in serum and reproductive tract flushings

Similarly to intradermal and intra-nasal immunization, intra-vaginal immunization also induced a high titer of HA specific IgG in serum and much lower titers of IgG in most flushing samples from all regions of the reproductive tract at both time points (Figure 4-7). In some of the flushing samples, especially some vaginal flushings, HA IgG was undetectable (Figure 4-7). As a consequence, the mean HA specific IgG titer in vaginal flushing was significantly lower ($P<0.05$) than that in oviductal flushing at 30 days post-infection.

Compared to the intra-nasal immunisation group (Figure 4-3), intra-vaginal infection induced a lower HA specific IgG response in serum at either 15 days ($P<0.05$) or 30 days ($P<0.05$) post-infection and a lower HA specific IgG response in oviductal

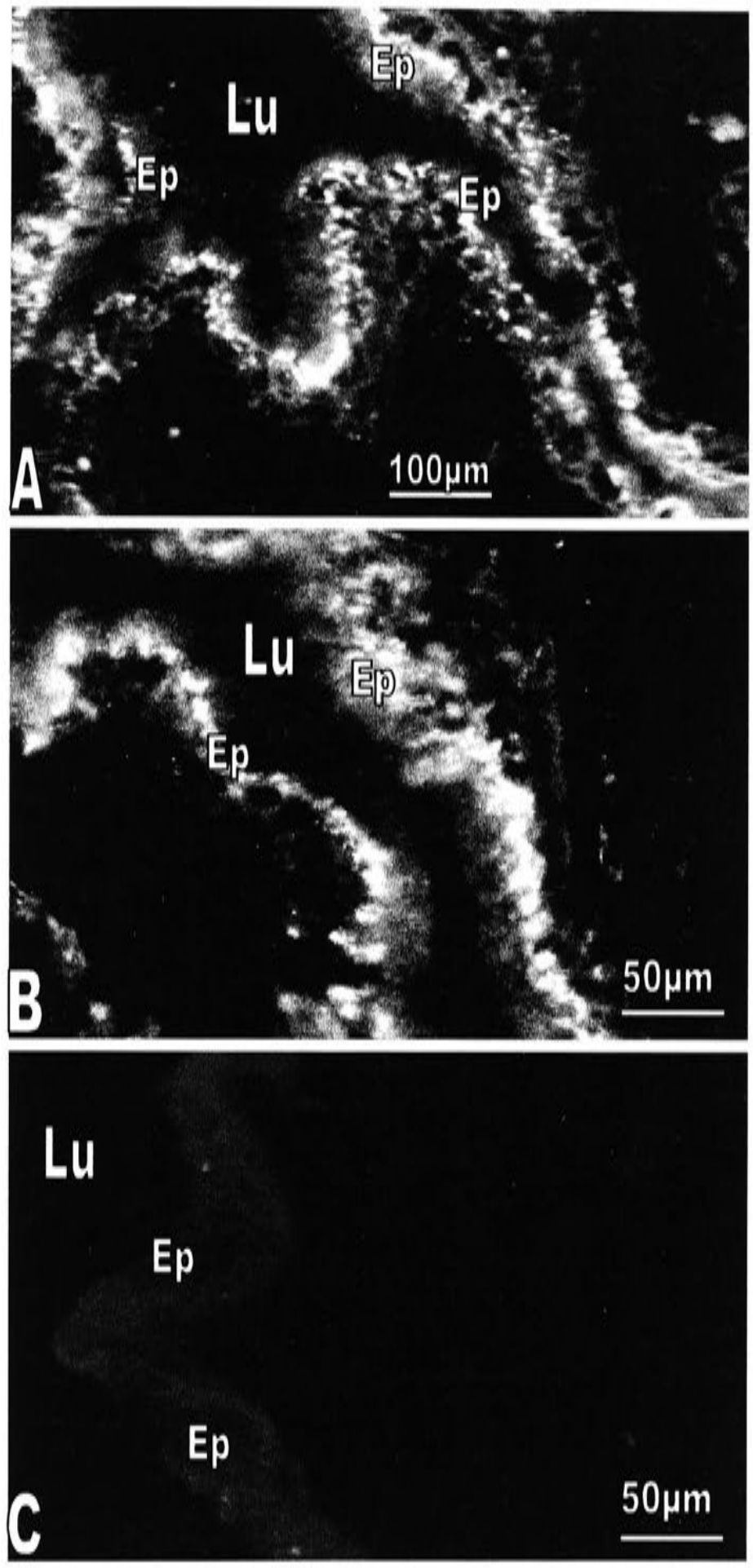


Figure 4- 5. The location of HA positive cells in the vagina of intra-vaginally immunised rabbits. Image A shows the location of HA positive cells in the vagina. Image B is a higher magnification of A. Positive cells are mainly present in the epithelium or the sub-epithelium immediate beneath the epithelium. This location was similar to that of MV positive cells shown in Figure 4-4A. Image C is vagina from same infected rabbit treated with normal rat serum. Lu: lumen; Ep: luminal epithelium.

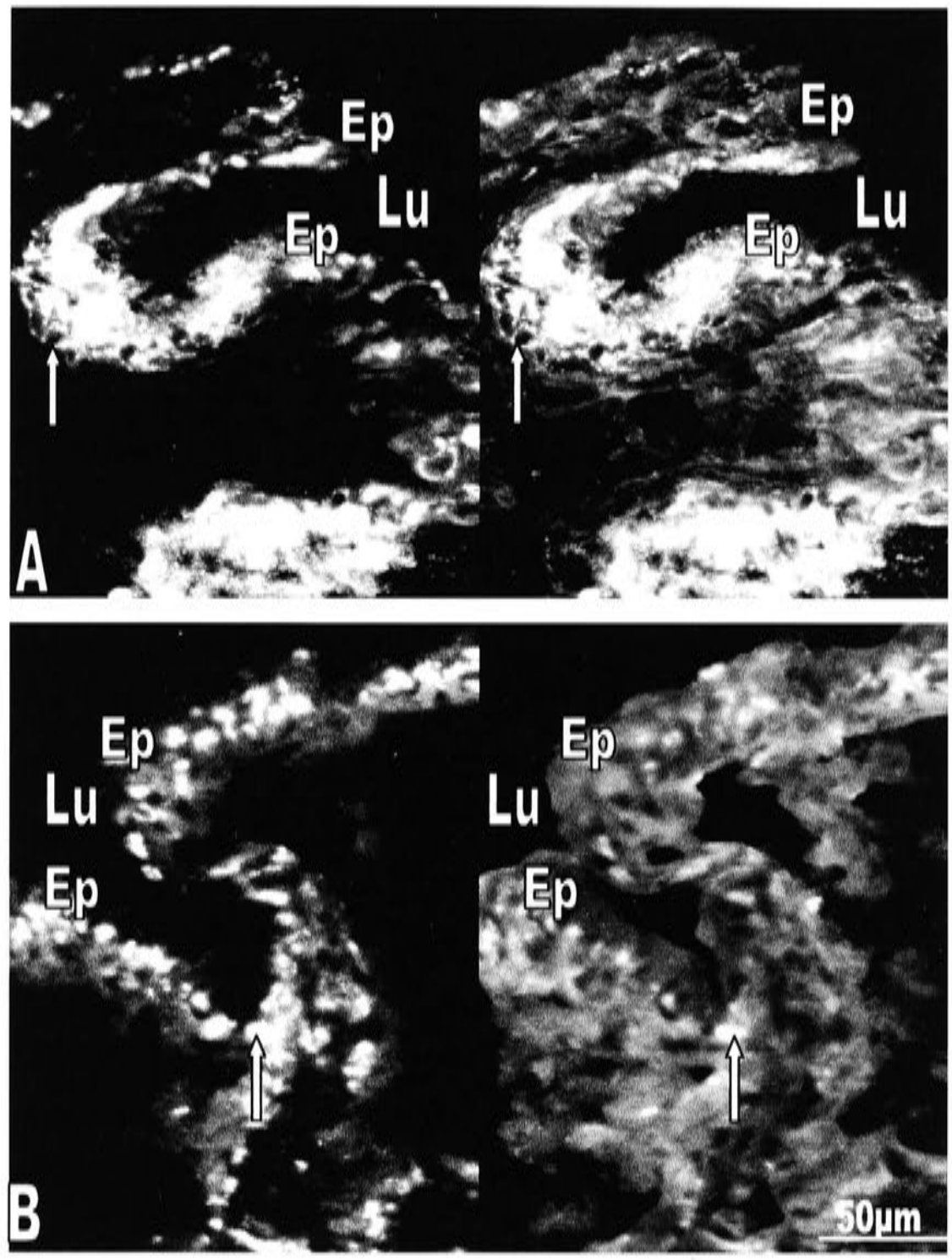


Figure 4- 6. Myxoma virus and MHC class II double staining in the lower reproductive tract of intra-vaginally immunised rabbits. The immunofluorescent images show double staining of MV and MHC II positive cells on the same sections. A is the vagina and B is the cervix. Each image contains two parts: the left part shows MV staining and the right part shows MHC II staining. Positive cells were located mainly in the epithelium. In both regions, most MV positive cells were also MHC class II positive (A and B, arrows). Lu: luminal epithelium; Ep: epithelium. Both images are at same magnification.

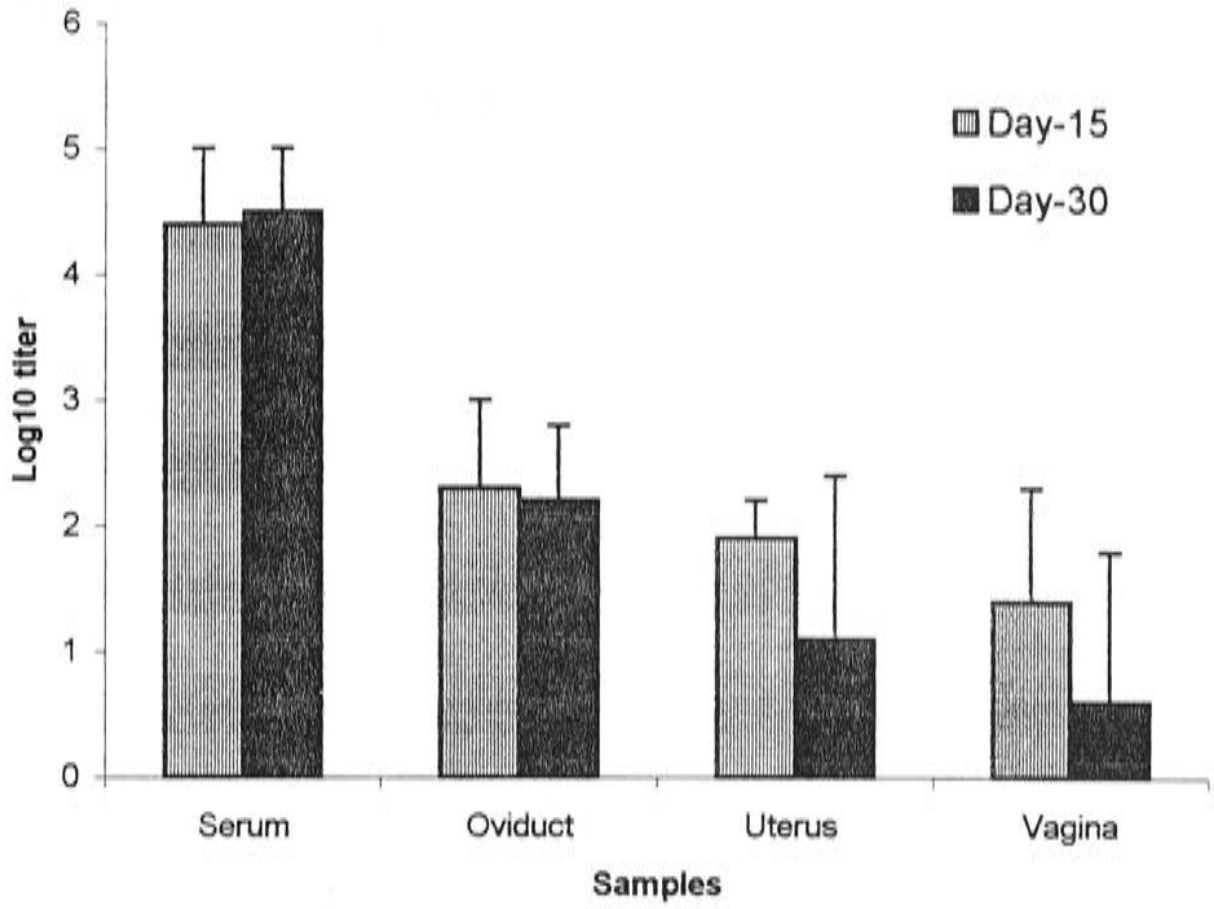


Figure 4- 7. HA specific IgG titers in serum, reproductive tract flushings and nasal washings after intra-vaginal immunisation

Rabbits were intra-vaginally immunised with 10^6 pfu MV-HA. IgG titers to HA were measured by ELISA in serum and flushings from oviduct, uterus and vagina at 15 and 30 days post-infection. The antibody titers were transformed into logarithms and expressed as mean Log_{10} titers. n = four rabbits.

($P<0.05$) and vaginal ($P<0.05$) flushings at 30 days post-infection. However, the HA antibody titers in the serum or reproductive tract flushings obtained after intra-vaginal immunisation were not significantly different from those following intradermal immunisation, except that the HA IgG titre in vaginal flushings was lower ($P<0.05$) at the 30 day time point. HA IgG was detected in only one nasal washing sample at a titer of 1:50, reflecting a poor IgG response in the nasal passage following intra-vaginal immunisation compared with intranasal immunisation. No HA specific IgG was detectable in serum, nasal washings or vaginal washings collected before infection except for one serum sample that showed some background reactivity. However, this was 60-fold lower than the titer after infection. MV antibody was not detectable in pre-immune serum of this rabbit but was present after infection.

HA IgM ranged from 1:25 to 1:1600 in serum and was higher ($P<0.05$) at 15 days (mean ± S.D, 2.6 ± 0.5) than 30 days (mean ± S.D, 1.8 ± 0.3) post-infection. However, HA IgM was detected only in oviductal flushings from 2 rabbits at a titer of 1:5 but was not detectable in other flushing samples, pre-immune serum or nasal and vaginal washings.

HA IgA was detected in one oviductal and one uterine flushing sample from different rabbits at a titer of 1:3 but was not detectable in serum or other flushing samples.

4.3.5 Antibody response to the vector, myxoma virus

Because HA was delivered by recombinant myxoma virus an antibody response was mounted not only to HA but also to myxoma virus itself. IgG and IgA to myxoma virus were measured in serum, reproductive tract flushings, and nasal washings from intra-nasally and intra-vaginally immunised rabbits using ELISA (Chapter 2 section 2.3.1).

A strong specific IgG response to myxoma virus was detected in serum (Table 4-1). MV specific IgG was also detectable in most reproductive tract flushings from the intra-nasal group and some flushings from the intra-vaginal group but the titers were much lower than those in serum (Table 4-1). Intra-nasal infection induced a higher MV specific IgG response in both serum and reproductive tract fluids compared to the intra-vaginal infection, which was similar to the IgG response to HA. MV IgA was not detectable in any of these samples by ELISA.

Table 4-1. Individual MV IgG titers in serum, reproductive tract flushings, and nasal washings after mucosal immunisations with MV-HA

Group	Time point	Serum	Oviduct	Uterus	Vagina	Nasal
Intra-nasal	Day 15	4.1, 4.1	1.3, 1.6	1.6, 1.3	1.0, 0	1.0, 1.0
	Day 30	4.7, 4.7	2.9, 2.9	2.0, 2.0	2.0, 1.3	1.6, 1.3
Intra-vaginal	Day 15	3.2, 2.6	1.6, 0	1.0, 0	0, 0	0, 0
	Day 30	3.5, 4.1	1.6, 1.3	1.3, 0	0, 0	1.0, 0

The values are Log_{10} titers.

4.3.6 Time course of antibody response after mucosal infection with MV-HA and intra-vaginal boost with HA

To extend the time-course of the study, two rabbits were intra-nasally and two intra-vaginally immunised with MV-HA. IgG and IgA to HA and MV in serum and vaginal washings were measured at 3, 15, 30, 45, and 70 days post-infection to determine whether there was a delayed antibody response. To investigate whether subsequent boosting would elevate IgA antibody production, the infected rabbits were then intra-vaginally boosted with influenza virus (HA) at 70 days post-infection. Specific IgG and IgA to HA were measured in serum and vaginal washings at day 3, 6, and 10 after boosting.

4.3.6.1 HA IgG and IgA response in serum and vaginal washings

High and lasting specific IgG responses to HA were induced in serum following immunisation via both routes (Figure 4-8). The titers of HA specific IgG in vaginal washings basically paralleled those in serum but at titres about 100-fold lower (Figure 4-8). Compared with the intra-vaginal route, intra-nasal immunisation stimulated a higher HA specific IgG response in both serum and vaginal washings. No anamnestic response was seen following boosting at day 70. Although antibody levels were elevated slightly 3-6 days after boosting, this was not a typical secondary antibody response.

HA IgA was not detectable in any serum or vaginal washing samples after immunisation by either intra-vaginal or intra-nasal routes followed by intra-vaginal boosting.

4.3.6.2 HA IgG and IgA in reproductive tract flushings after boost

The boosted rabbits were killed 15 days after the boost and blood and reproductive tract flushings from each region were collected for antibody measurement. Compared with serum, much lower HA specific IgG was detected in reproductive tract flushings (Table 4-2). HA specific IgA was not detectable in any of these samples.

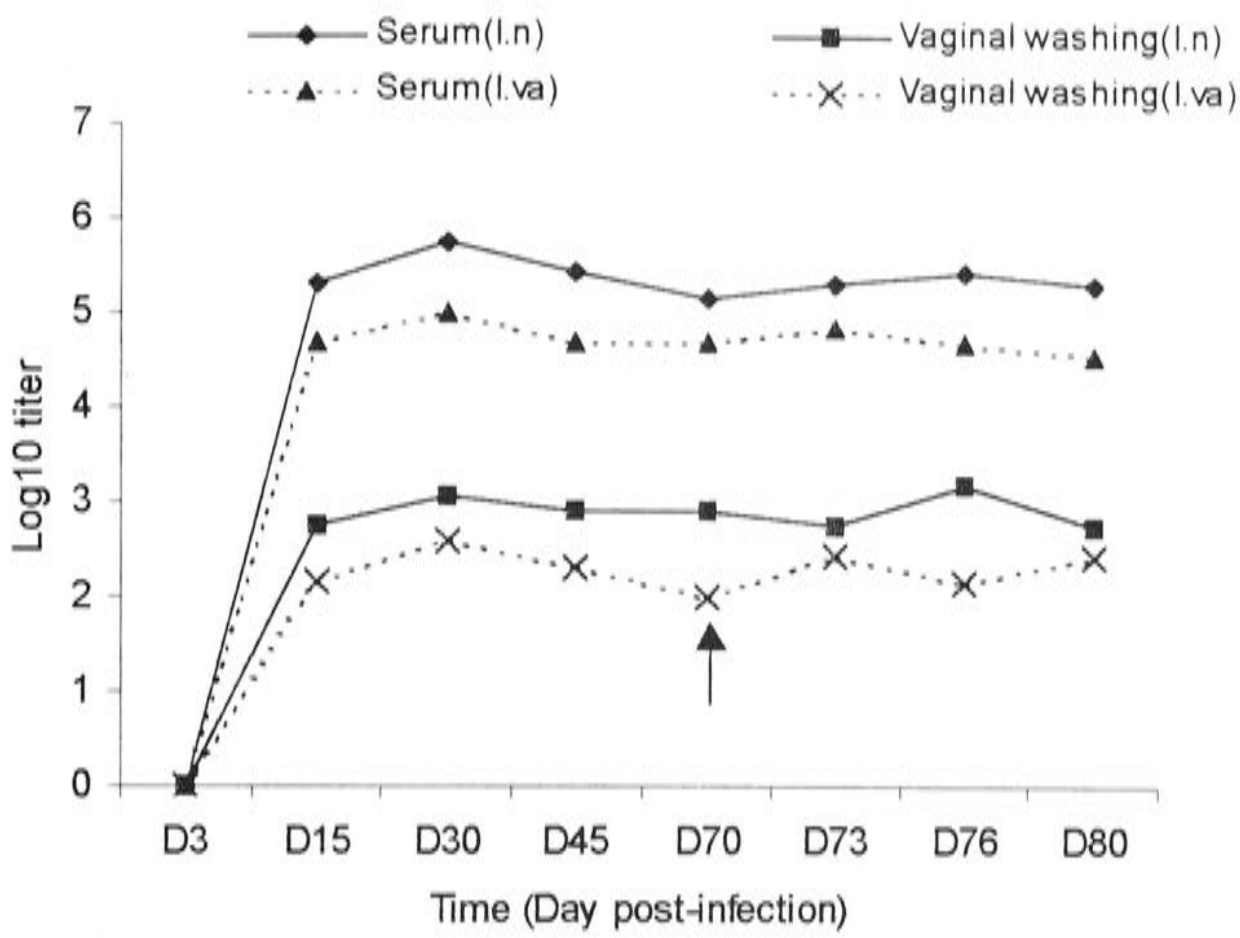


Figure 4- 8. HA specific IgG titers in serum and vaginal washings after mucosal infection with MV-HA and intra-vaginal boost with HA

Rabbits were intra-nasally (I.n) or intra-vaginally (I.va) infected with 10^6 pfu MV-HA followed by an intra-vaginal boost (arrow) with influenza virus (HA) at 70 days post- infection. HA IgG titers were determined by ELISA in serum and vaginal washings collected at day 3, 15, 30, 45, 70 post-infection and 3, 6, 10 post-boosting. The antibody titers were transformed into logarithms and expressed as Log_{10} titers (mean of two rabbits).

Table 4- 2. HA specific IgG titers in serum and reproductive tract flushings after infection with MV-HA and 15 days after boosting with HA

Routes	Serum	Oviduct	Uterus	Vagina
Intra-nasal	5.3, 5.0	2.6, 2.3	2.6, 2.3	2.3, 2.3
Intra-vaginal	4.7, 4.4	2.0, 1.7	1.7, 1.4	2.3, 2.0

The values are Log_{10} titers.

4.3.6.3 MV specific IgG and IgA in vaginal washings

IgG and IgA specific to the vector, myxoma virus, were also measured in serum and vaginal washings over the time course experiment. MV IgG titers were high in serum

and much lower in vaginal washings (Table 4-3). No MV IgG was detectable in serum collected before immunisation. IgA to MV was undetectable in either serum or the tract fluids before or after immunisation.

Table 4- 3. MV IgG titers in serum and vaginal washings in a time course after infection with MV-HA

Groups	15 days PI		30 days PI		45 days PI	
	Serum	Vagina	Serum	Vagina	Serum	Vagina
Intra-nasal	4.1, 3.8	2.0, 1.6	4.7, 4.7	2.3, 1.0	4.7, 4.4	2.0, 1.0
Intra-vaginal	3.5, 3.5	1.6, 1.3	4.1, 3.8	1.6, 1.3	3.8, 3.5	2.0, 1.0

The values are Log_{10} titers. PI: post-infection with MV-HA.

4.3.7 Antibody production in the reproductive tract

4.3.7.1 Total IgA titers in serum and reproductive tract fluids

If IgA plasma cells specific for HA homed back to the reproductive tract after mucosal immunisation and contributed to local IgA production, an elevated total IgA level might be expected in the luminal fluids. To test this, total IgA titers were measured by immunodiffusion as an alternative to ELISA (Chapter 2 section 2.3.2.2).

In intradermally infected or uninfected control groups, total IgA titers in serum were about 100 fold higher than that in reproductive tract fluids and IgA was undetectable in about half the fluid samples (Table 4-4). Compared with normal rabbits, induction of ovulation (hCG treatment) and route of infection did not significantly change the total IgA titers in either serum or reproductive tract fluids. No differences in total IgA titer could be seen between the samples from different regions of the reproductive tract or between the samples collected by flushing or micropuncture (Table 4-4). After mucosal immunisation, the total IgA titers in reproductive tract flushings were not significantly

Table 4- 4. Total IgA titers in serum and reproductive tract fluids of control and intradermally immunised rabbits

Groups	Serum	Flushings			Micropunctures		
		Oviduct	Uterus	Vagina	Oviduct	Uterus	Vagina.
Controls	256, 128	1, 0, 0, 0	2, 1, 1, 0	1, 0, 0, 0	nd	nd	nd
HCG	258, 128	4, 2, 0, 0	1, 1, 0, 0	8, 0, 0, 0	1, 0	2, 0	2, 1
Day 15	128, 64	2, 1, 0, 0	2, 1, 1, 0	1, 0, 0, 0	1, 0	1, 1	2, 0
Day 15 +hCG	256, 256	1, 0, 0, 0	1, 0, 0, 0	2, 1, 0, 0	1 [a]	2 [a]	4, 0
Day 30	256, 256	8, 1, 1, 0	4, 1, 1, 0	1, 1, 0, 0	0, 0	2, 0	2, 1
Day 30 +hCG	256, 128	4, 1, 0, 0	2, 1, 0, 0	0, 0, 0, 0	1, 0	1, 0	8, 1

The values are reciprocal titers of the last dilution in a two-fold serial dilution, giving a positive result. 1: means without dilution of the sample; 0: means no precipitate band. HCG: hCG treated controls; Day 15, Day 30: 15 or 30 days post-infection with MV-HA; + hCG: infection with induction of ovulation. nd: not measured; a: the sample is not available because the rabbit died before sampling.

elevated at either 15 or 30 days compared to controls and the intra-dermal group (Table-4-5). Total IgA titers in nasal washings from both mucosal immunisation groups were also measured and were not different from reproductive tract flushings (Table 4-5).

4.3.7.2 HA specific antibody producing cells in reproductive tract tissues

To further examine whether plasma cells in the reproductive tract were HA antibody-specific cells, influenza virus was used to bind to HA antibody in these cells. Virus binding was detected by rat anti-HA followed by anti-rat FITC on frozen sections from various regions of the reproductive tract (Chapter 2 section 2.4.2). Popliteal lymph nodes draining the inoculation site of intradermally infected rabbits or from uninfected rabbits were also labelled as positive or negative controls. This assay had a considerable background which shows as punctate staining in Figure 4-9. This background might be due to influenza virus binding to sialic acid residues on the cell surface; sialic acid is

Table 4- 5. Total IgA titers in serum and reproductive fluid samples after mucosal immunisations with MV-HA

Groups		Day 0 serum	Day 15/30 Serum	Oviduct	Uterus	Vagina	Nasal
Intra-nasal	Day 15	128, 64, 64, 64	128, 64, 64, 128	1, 0, 1, 0	1, 0, 1, 0	1, 0, 0, 1	0, 0, 1, 1
	Day 30	256, 256, 128, 128	256, 512, 128, 128	4, 2, 1, 1	0, 0, 0, 0	0, 0, 0, 0	1, 2, 0, 0
Intra-vaginal	Day 15	128, 64, 128, 512	128, 64, 64, 256	1, 1, 1, 4	0, 0, 0, 1	0, 0, 0,0	0, 0, 1, 0
	Day 30	256, 64, 256, 128	64, 128, 256, 256	1, 4, 1, 1	1, 0, 0, 0	0, 0, 0, 0	0, 0, 0, 0

The values are reciprocal titers of the last dilution in a two-fold serial dilution giving a positive result. 1: means no dilution of the sample; 0: means no precipitate band.

the normal receptor for influenza virus. Nevertheless, the strong signal of the antibody producing cells can be easily identified in Figure 4-9. This assay also included anti-sera controls (normal rat serum instead of rat anti-HA serum) and antigen controls (omit influenza virus antigen). This second control is extremely important because anti-HA serum can also detect virus-infected cells that are producing HA protein.

Whereas positive cells were frequently seen in the sections of popliteal lymph nodes from the intradermally infected rabbits, positive cells were rarely found in the sections of reproductive tract tissues from the same infected rabbits at 30 days post-infection (Figure 4-9). Even in serial sections of the uterus from several intra-dermally immunised rabbits only a few positive cells were found (data not shown). Some vaginal and cervical sections contained a few more positive cells than uterine sections. No positive cells were found in reproductive tract sections from uninfected controls or from the negative controls. Induction of ovulation by HCG treatment did not affect the number of HA antibody-producing cells.

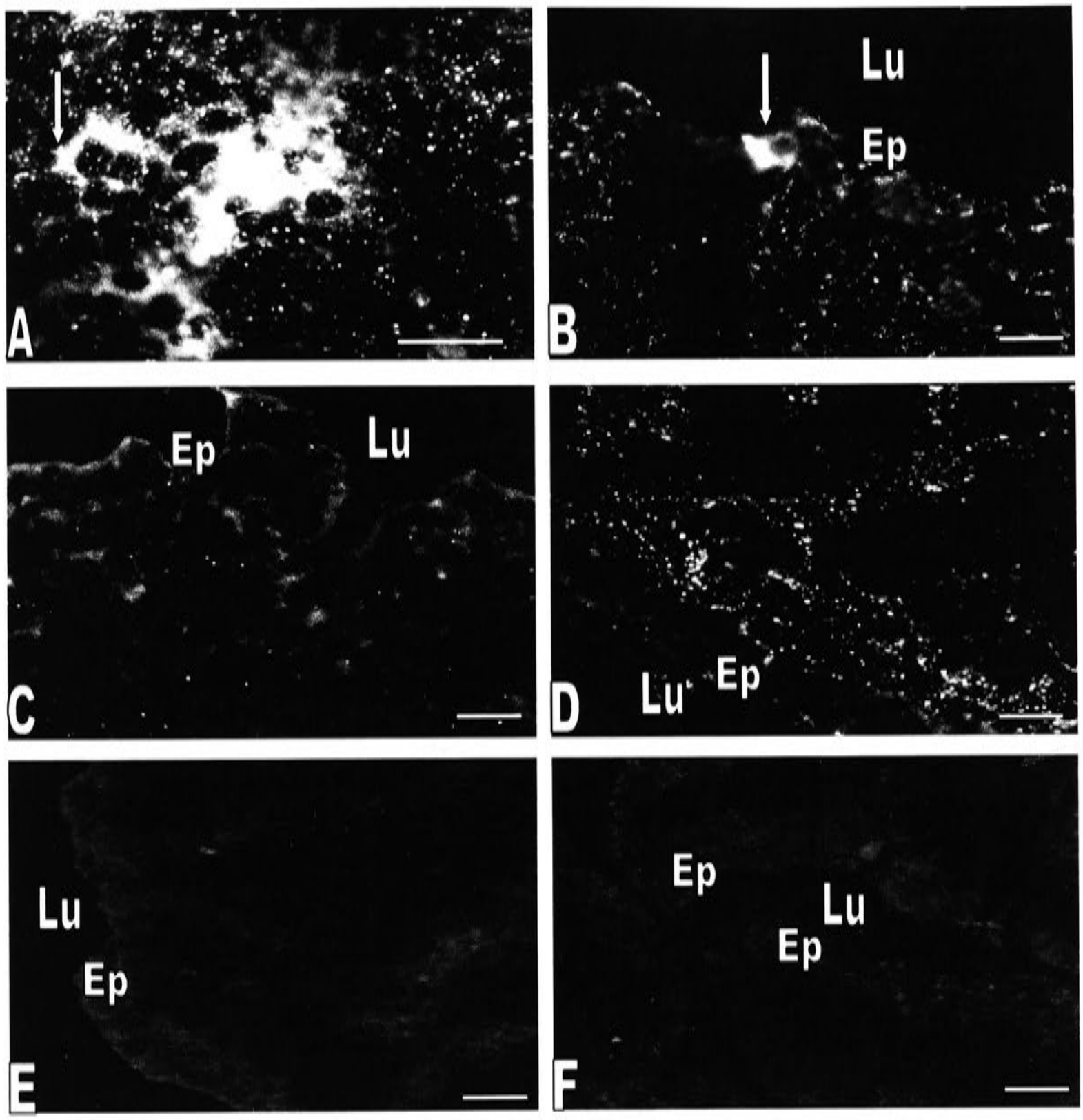


Figure 4-9. HA specific antibody secreting cells in popliteal lymph nodes and the uterus of MV-HA infected rabbits. The immunofluorescent images show that many HA specific antibody producing cells were present in the lymph node (A, arrow) draining the inoculation site whereas only one was found in whole uterine section (B, arrow) from the same rabbit. Image C shows no positive cells were seen in the negative control with normal rat serum replacing rat anti-HA serum on an uterine section from an infected rabbit. Image D shows an uterine section from an uninfected rabbit stained for HA antibody producing cells. E and F show the controls omitting influenza virus in the uterus and cervix, respectively. Lu: lumen; Ep: luminal epithelium. The scale bars represent 25 micro-meters.

Following intra-nasal immunisation, positive B cells were occasionally seen in the sub-epithelium of the oviduct and uterus and a few positive cells were observed in the epithelium of the cervix from some rabbits. This was similar to figure 4-9b (data not shown). In the intra-vaginal group, the virus replicated in vagina and cervix and HA was expressed in these tissues (section 4.3.4.1). This interfered with the staining of HA specific antibody producing cells in the vagina and the cervix. Nevertheless, positive cells were occasionally found in the oviduct and uterus of some rabbits in this group (data not shown). These results suggest that the local production of HA specific antibody is of limited importance.

4.3.7.3 Source of IgG in reproductive tract fluids

Low titers of HA specific IgG were present in the reproductive tract fluids after either intradermal or mucosal immunisation with MV-HA. However, as shown above, very few HA antibody-secreting cells were present in the reproductive tract which suggests that HA antibody in the reproductive tract must be derived predominantly from serum. To test this further and to examine the effect of ovulation on IgG titer in reproductive tract fluids, the total IgG titers in serum and reproductive tract fluids were measured using ELISA (Chapter 2 section 2.3.2.1) and the ratio of total IgG and HA specific IgG was calculated. While the total IgG and the HA IgG ELISA titers are not necessarily comparable, this does allow a comparison to be made between compartments and treatments within the experiment.

In all control and intradermal immunised groups, the total IgG titers in serum were more than 100 fold higher than those in reproductive tract fluids (Table 4-6 and 4-7). Induction of ovulation did not significantly change total IgG titers in either serum or reproductive tract flushings. No significant differences were found between samples collected from different regions for either immunised or unimmunised groups (Table 4-6). The total IgG titers obtained in flushing samples were not significantly different from those obtained in micro-puncture samples though they were generally slightly lower (Table 4-7).

After mucosal immunisation, the total IgG titers in serum were more than 100 fold higher than those in reproductive tract flushings at either day 15 or day 30 (Table 4-8).

Table 4- 6. Total IgG titers in serum and reproductive tract flushings in intradermal infected and uninfected control rabbits

Groups		Serum	Oviduct	Uterus	Vagina
Controls		4.9 ± 0.2	2.5 ± 0.3	2.7 ± 0.3	2.1 ± 0.3
hCG		4.8 ± 0.2	2.7 ± 0.3	2.5 ± 0.4	2.4 ± 0.7
MV-HA	Day 15	4.8 ± 0.3	2.5 ± 0.4	2.3 ± 0.7	2.3 ± 0.5
	Day 30	4.8 ± 0.2	2.5 ± 0.6	2.6 ± 0.4	2.2 ± 0.5
MV-HA+ hCG	Day 15	5.0 ± 0.3[a]	2.4 ± 0.3	2.5 ± 0.3	2.4 ± 0.6
	Day 30	4.9 ± 0.3	2.8 ± 0.4	2.3 ± 0.4	2.2 ± 0.4

The values represent mean Log_{10} titers ± S.D for four rabbits. hCG: hCG treated controls; +hCG: infection with induced ovulation. a: One rabbit dies so only three samples were available.

Table 4- 7. Total IgG titers in micropuncture samples of reproductive tract from intradermal infected and uninfected control rabbits

Groups		Oviduct	Uterus	Vagina
HCG		2.9, 2.6	2.9, 2.6	3.2, 2.9
MV-HA	Day 15	2.9, 1.7	2.3, 2.3	2.3, 2.0
	Day 30	2.6, 2.3	2.9, 2.6	2.9, 2.9
MV-HA+ HCG	Day 15	3.2[a]	2.9[a]	2.9, 2.6
	Day 30	2.3, 2.0	2.9, 2.6	2.9, 2.6

The values are Log_{10} titers. a: One rabbit was dead before sampling.

In the intra-nasal immunised group, the mean IgG titers in oviduct were higher than those in uterine ($P<0.01$) or vaginal ($P<0.001$) flushings at 30 days post-infection (Table 4-8). In the intra-vaginal immunised group, total IgG titers in the oviduct were higher than those in uterine ($P<0.01$) or vaginal ($P<0.05$) flushings at 15 days post-infection and higher than those in vaginal flushings ($P<0.05$) at 30 days post-infection (Table 4-8). However, no differences of total Ig titers were found between the intranasal and intra-vaginal groups except that the total IgG titer in vaginal flushings of the intra-vaginal group was lower ($P<0.05$) than that of intra-nasal group. In addition, total IgG titers in uterine flushings of the intranasal group at 30 days post-infection and total IgG

Table 4- 8. Total IgG titer in serum and reproductive tract flushings after mucosal immunisations

Groups		Day 0 serum	Day 15/30 serum	Oviduct	Uterus	Vagina
Intra-nasal	Day 15	4.9 ± 0.2	5.2 ± 0.3	2.8 ± 0.4	2.6 ± 0.4	2.4 ± 0.4
	Day 30	4.7 ± 0	5.2 ± 0.2	2.8 ± 0.2	1.7 ± 0.5	1.9 ± 0.2
Intra-vaginal	Day 15	4.9±0.3	4.9 ± 0.2	3.1 ± 0.3	2.1 ± 0.4	2.0 ± 0.7
	Day 30	4.7 ± 0	5.0 ± 0.2	2.2 ± 0.5	1.1 ± 1.3	0.8 ± 0.9

The values are mean Log_{10} titers ± S.D for four rabbits.

titers in vaginal flushings of the intra-vaginal at 30 days post-infection were lower ($P<0.05$ for both) than those in the uninfected control group.

For most MV-HA infected groups, the ratio of total IgG in serum to that in the reproductive tract fluids was similar to the that of HA IgG in these compartments (Table 4-9). The similarity in these ratios suggested that the HA specific IgG in the reproductive tract fluids was mainly derived from serum and that local antibody production did not contribute significantly. However, in the intra-vaginal group, the ratio of specific IgG was higher than that for total IgG at both 15 and 30 days post-infection. In addition, the ratios of specific IgG were generally a little higher at 15 days than at 30 days, suggesting that, if there were a small amount of local IgG production, it might occur at around 15 days post-infection.

4.3.8 Antibodies to HA in serum and ovarian follicular fluids following MV-HA infection

Ovarian fluid is derived from serum and might therefore be expected to have similar antibody titers to serum. To verify if serum antibodies do indeed freely transfer into ovarian follicles, rabbits were intradermally infected with MV-HA and HA specific

Table 4- 9. The ratios of total IgG and HA-IgG in serum and reproductive tract flushings from rabbits immunised with MV-HA through different routes.

Immunisation routes and days of sampling		Oviduct total IgG	Oviduct HA-IgG	Uterus total IgG	Uterus HA-IgG	Vagina total IgG	Vagina HA-IgG
Intra-dermal	Day 15	1.9	2.3	2.1	2.6	2.1	2.3
	Day 30	1.9	2.0	1.8	1.8	2.2	2.2
Intra-dermal + hCG	Day 15	2.1	2.3	2.0	2.4	2.1	2.5
	Day 30	1.8	1.8	2.1	2.1	2.2	2.2
Intra-nasal	Day 15	1.9	2.1	2.0	2.4	2.2	2.6
	Day 30	1.9	1.7	3.1	2.0	2.7	2.3
Intra-vaginal	Day 15	1.6	1.9	2.3	2.3	2.5	**3.1**
	Day 30	2.3	2.0	4.5	4.1	6.3	**7.5**

The values are ratios of mean IgG titers for four rabbits.

antibody levels in serum and ovarian follicular fluids were measured at 15 days post-infection.

After super-ovulation, three of the 4 infected rabbits developed very good pre-ovulatory follicles. Follicular fluids from all four rabbits were collected as described in Chapter 2 (section 2.2.3.1) and pooled for each rabbit. Generally, 20-50 µl was collected from each ovary. HA specific IgG, IgM and IgA in serum and ovarian follicular fluids were measured using ELISA (Chapter 2 section 2.3.1).

There were no differences in the titers of HA IgG or IgM in ovarian follicular fluids and serum of the four immunised rabbits (Figure 4-10). Specific IgA to HA was not detectable in either serum or ovarian follicular fluids. This result suggests that the anti-HA immunoglobulins in serum induced by recombinant myxoma virus can freely transfer into ovarian follicle fluids. This implies that if high antibody titers are required for immunocontraception, then the ovarian follicle may be a more appropriate target than the mucosal compartments of the reproductive tract.

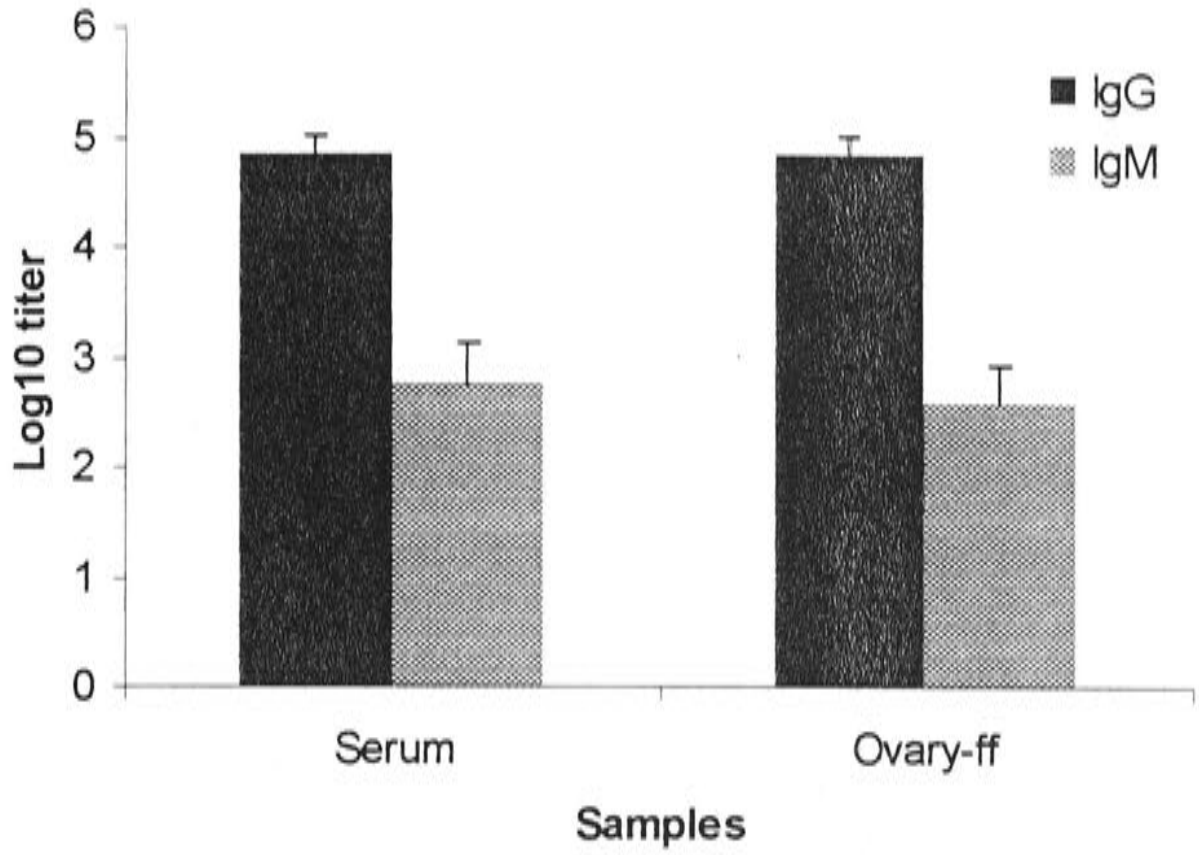


Figure 4- 10. HA IgG and IgM titer in serum and ovarian follicular fluids.

Rabbits were infected with MV-HA by the intradermal route. HA titers of IgG and IgM were measured in serum and ovarian follicle fluids (ovary-ff) at 15 days post-infection using ELISA. Antibody titers were transformed into logarithms and expressed as Log_{10} titer. The bars represent means of four rabbits and error bars are S.D.

4.4 Discussion

4.4.1 Is myxoma virus an efficient vector for inducing a mucosal antibody response in the female reproductive tract?

4.4.1.1 Immunisation through intradermal inoculation

The ability of antigens to induce a strong, specific antibody response within the female reproductive tract is crucial to the success of immunocontraceptive vaccines which seek to act through prevention of fertilisation (Chapter 1 section 1.2.2). In this chapter, the antibody response in serum and within the different regions of the female rabbit reproductive tract to the influenza HA delivered by a recombinant myxoma virus

was investigated as a model for a virus delivering a reproductive antigen. Several different routes of administration were compared for their capacity to induce a sustained antibody response within the tract. Measures were made in both sexually quiescent animals and in animals within 12 hours of ovulation.

Kerr and Jackson (1995) first constructed and described the recombinant virus used in this study. They showed that as little as 5 pfu was sufficient to infect the animals and induce an immune response to the HA antigen, despite the fact that insertion of the HA gene resulted in attenuation of the virus compared to the parental strain. The recombinant virus did not cause significant clinical disease in domestic rabbits making it an ideal model to study the properties required of an immunocontraceptive vector. In addition, HA is a well-characterised, highly immunogenic protein which is not present in the female reproductive tract hence making the antibody response easy to measure. Indeed in Kerr and Jackson's original study, HA specific antibody was present in vaginal fluids as well as plasma. In our initial experiment we sought to confirm and extend these results to the oviduct, uterus and cervix.

Following intradermal immunisation with MV-HA, HA antibody titers were induced in serum by day 15 post-infection and they remained elevated at 30 days post-infection. However, IgG antibody titers in the fluids of the oviduct, uterus and vagina were only 0.1-1% of those detected in serum. Moreover, IgA to HA was not detectable in either serum or reproductive tract fluids after the infection. Titers of IgG and IgA to HA in reproductive tract fluids were not significantly influenced by ovulation. These results are in sharp contrast to a number of studies in rodents (Wira and Sandone, 1987; Gallichan and Rosenthal, 1995; 1996; Johansson *et al.* 1998). In these studies, specific IgG antibody levels in reproductive tract fluids were about 10% of the serum level in mice (Gallichan and Rosenthal, 1995; Johansson *et al.* 1998). IgA antibody levels increased in female genital secretions after oestradiol treatment in mice (Wang *et al.* 1996) and rats (Wira and Sandone, 1987), whereas IgG antibody titers in vaginal washes were significantly higher at the dioestrus stage or after progesterone administration (Gallichan and Rosenthal, 1996). These findings indicate that, in rodents at least, IgG and IgA vary cyclically through the oestrous cycle with IgA being high when oestrogen levels are high and IgG being elevated when progesterone dominates. These cyclical changes in immunoglobulin concentration are probably associated with

the protection of the reproductive tract from external infections and with facilitating the implantation of the conceptus. However, unlike rodents, the rabbit does not have an oestrous cycle being held in prooestrus under the control of oestrogen until ovulation is stimulated by mating and it is probably not surprising that there are differences in the immune response between rodents and rabbits.

These results suggest that, by the natural infection route, the recombinant virus is unable to induce a high-level antibody response in the reproductive tract fluids of female rabbits under either oestrogen domination or post ovulation. In particular, IgA to HA could not be induced. Data from a previous study in rabbits also demonstrated that systemic immunisation was not effective at inducing an IgA response in the female reproductive tract to a model antigen horse-radish peroxidase (HRP) compared with local immunisation routes though IgG antibody was induced in serum by both systemic and local immunisations (McAnulty and Morton, 1978). The reason for this may be because Ig switching to IgA isotype occurs mainly in mucosal tissues such as Peyer's patches (Spieker-Polet *et al.* 1993) and not in other lymphoid organs. Obviously, if recombinant myxoma virus is to be used as an immunocontraceptive vaccine vector alternative ways of antigen presentation need to be considered if an antibody response in the reproductive tract is necessary.

4.4.1.2 Immunisation via mucosal routes

Antigen presentation through common mucosal inductive sites and local antigen presentation through the vaginal mucosa are considered to be effective ways to stimulate mucosal antibody responses in the female reproductive tract (Chapter 1 section 1.5.1 and 1.5.2). To be able to maximise the antibody response in the reproductive tract and induce an IgA response, administration of MV-HA through a distal mucosal site, the nasal mucosa, and a local mucosal site, the vaginal mucosa, was tested. Both the inflammatory response and direct staining indicated that the recombinant virus replicated locally following the infection. However, antibody titers to HA in the reproductive tract were essentially similar to those following intradermal immunisation. HA IgA was not detectable in serum and only present in trace amounts in a few cases in reproductive tract fluids. These findings differ from those obtained in murine studies following immunization with a recombinant adenovirus

(Gallichan *et al.* 1993; Gallichan and Rosenthal, 1995; Xiang and Ertl, 1999). In these studies, specific IgA and IgG were induced in both serum and vaginal secretions to either HSV-1 glycoprotein B (Gallichan *et al.* 1993; Gallichan and Rosenthal, 1995) or rabies virus glycoprotein (Xiang and Ertl, 1999) following intra-nasal immunisation. Therefore, compared with recombinant adenovirus in the mouse, recombinant myxoma virus is much less efficient at inducing an IgA antibody response in the rabbit. However, it is unclear whether rabbits would mount a good IgA response to an antigen presented by an adenovirus because the appropriate experiment has not been done.

The antibody response to the vector, myxoma virus, was also assayed in serum and reproductive tract fluids following mucosal immunisation with MV-HA. The antibody responses to MV were similar in timing and titer to those to HA and no IgA response to MV was detected. This result suggests that MV itself is not effective at stimulating an IgA response. The inability of the recombinant myxoma virus to provoke an IgA response systemically or locally to either the vectored antigen HA or to the virus itself makes it a poor delivery vehicle for induction of an effective IgA response within the reproductive tract. This inefficiency may relate to its immuno-suppressive effect. MV produces several homologues of Th1 cytokine receptors (such as TNF-αR, INF-γR) that will interfere with cytokine networks (McFadden *et al.* 1995). MV infection also down regulates MHC-I molecules on the surfaces of infected cells (Nash et al. 1999; Guerin *et al.* 2002) which will inhibit antigen presentation to cytotoxic T-lymphocytes. Myxoma virus also specifically down-regulated the CD4 molecule on the surface of infected cells by degrading CD4 in lysosomal vesicles (Barry *et al.* 1995). This would result in the inhibition of recognition by these cells of peptides presented by MHC-II on APC or B cells thus affecting activation of T cells and potentially the generation of cellular and humoral immune responses. The IgA response has been reported to be highly dependent on CD4+ T helper (Th) cells and Th2 cytokines such as IL-4, 5, 6 and 10 are essential in IgA isotype-switching and IgA production (Husband *et al.* 1994). The viral infection of CD4+ cells and down-regulation of CD4 molecules on CD4+ T cells may thus disturb the production of these Th2 cytokines and hence IgA isotype-switching and production. A recent study showed that co-expression of IL-5 and 6 within a recombinant adenovirus could enhance the IgA response and IgA antibody production in the lung following intra-nasal infection (Braciak et al. 2000). This result supports the view that Th2 cytokines are important in IgA antibody response. This indicates a potential

strategy for enhancing the mucosal response to myxoma virus but may not be relevant following intradermal inoculation because the virus is so attenuated that it is unlikely to spread to mucosal sites.

The data above collectively suggest that recombinant MV is not an effective vector for inducing a high-titer IgA or IgG antibody response in the female rabbit reproductive tract. If a reproductive antigen gene replaced the HA gene in the recombinant virus a lower antibody response in the reproductive tract would be anticipated because such antigens are usually less immunogenic than HA. This implies that without further modification of the recombinant myxoma virus it is not suitable to deliver reproductive antigens, such as sperm or oocyte antigens because it will not induce a high antibody titer within the reproductive tract that is capable of blocking fertilisation.

In the current study, the intra-vaginal route was less efficient at inducing systemic and local antibody responses compared with intra-nasal immunization. This was indicated by lower levels of HA IgG in serum and fewer reproductive tract fluid samples being positive for HA IgA. This result is consistent with many studies in both mouse (Gallichan and Rosenthal, 1995; Wu et al. 2000; Russell, 2002) and human (Kozlowski et al. 2002). For example, in mice, intra-vaginal immunisation with adenovirus expressing HSV-1 glycoprotein B resulted in a lower serum IgG and vaginal IgA antibody response than that following intra-nasal immunisation (Gallichan and Rosenthal, 1995). In humans, intra-vaginal immunization with 1 mg recombinant CTB (cholera toxic sub-unit B) was necessary to induce an IgA antibody response comparable to that generated by intra-nasal immunization with only 0.1mg recombinant CTB (Kozlowski et al. 2002). These results suggest that the vaginal mucosa is less effective than the nasal mucosa at mounting an immune response. This inefficiency of the vaginal mucosa could be explained in many ways. One possibility is the fact that compared with nasal mucosa, the female reproductive tract (including that of rabbits) lacks mucosal inductive site follicle-associated epithelium, M cells, and organized lymphoid tissues (McGhee et al. 1999). Direct antigen transport and antigen presentation, similar to that in the bronchus, could not occur in the vagina. The APC in the vagina must carry antigen (peptide) and migrate to local draining lymph nodes to initiate an immune response (McGhee et al. 1994). This suggests that relatively less

antigen presentation and a weaker immune response was mounted following intra-vaginal immunisation, with fewer B cells homing back to the reproductive tract.

Another possibility is the immunosuppressive effect of oestradiol on antigen presentation. Wira and Rossoll (1995a) showed that high levels of oestradiol administered to ovariectomised rats could inhibit the ability of vaginal APC to present antigen to T cells and that this inhibitory effect was reversed when progesterone was given along with oestradiol. The rabbit vagina is always under the control of oestradiol before mating, and so when MV-HA was administered locally into the vaginal mucosa, oestradiol might have inhibited antigen presentation by APC in the vagina and led to the lower antibody response. However, it remains to be determined if intra-vaginal administration of MV-HA after ovulation, a very artificial circumstance, could induce a stronger antibody response in rabbits.

4.4.2 Antibody response and transfer in the reproductive tract of female rabbits

4.4.2.1 IgA transfer in the reproductive tract

Induction of ovulation might increase IgA transudation from serum into the tissues in the oviduct and vagina (Chapter 3, section 3.3.7.2). But this was not reflected by the changes of general IgA titers in the luminal fluids. One possible explanation for this is that IgA might adhere tightly to the surface of the luminal epithelium or might even be within the epithelial cells, and not be efficiently transferred into the luminal fluids. This explanation is consistent with the observation of strong epithelial IgA staining along the luminal epithelial surface of the uterus and some cervices from both infected and uninfected rabbits. This phenomenon has not been reported in other species, so it is postulated that it could relate to the oestrogen domination in the reproductive tract of female rabbits.

The rabbit has 13 IgA constant region heavy chain genes (Spieker-Polet *et al.* 1993) whereas mice and human have only one or two isotypes. Furthermore, in the rabbit, different tissues express different IgA isotypes. For example, Cα4 mRNA is only

expressed in lung and spleen whereas Cα1, 6, 9, 10 and 12 are expressed in small intestine, mammary gland, salivary gland and appendix (Spieker-Polet *et al.* 1993). Among those isotypes, some are expressed at high levels, others at low levels (Spieker-Polet *et al.* 2002). It is not known how many isotypes are expressed in the reproductive tract or whether the anti-IgA antibody used in the present study, which was raised against pooled serum IgA, could measure all isotypes. Although it would be interesting to study IgA heavy chain expression in different tissues, such studies were beyond the scope of this thesis.

Previous studies showed that IgA in the epithelial cells or lamina propria could neutralise viruses and function locally (Kaetzel *et al.* 1991; Mazanec *et al.* 1992, 1993). In rabbits, the IgA on the epithelial surface or in the epithelial cells might act in the same way.

4.4.2.2 IgG antibody transfer in the reproductive tract

The IgG antibody present in female reproductive tract fluids is considered to derive mainly from serum (Parr and Parr, 1996). This is also true in rabbits in the current study as suggested by the observation that the ratios of specific and total IgGs in the reproductive tract fluids to those in serum were very similar. But local IgG production in the vagina may contribute a small amount following intra-vaginal immunisation. This is consistent with the result of the presence of IgG plasma cells in the vagina (Chapter 3). However, the IgG antibody titers in serum were about two orders of magnitude higher than those in the reproductive tract fluids following any immunisation procedures. These findings are consistent with other studies in rabbits and contrast with findings in the mouse. For example, IgG antibody was either not detected at all in the female rabbit reproductive tract (Edwards, 1960; Shapiro, 1971) or was detected in oviductal fluids at only 4 % of the levels in serum (Oliphant *et al.* 1977). Similarly, in oviductal fluids of rabbits immunised with sperm protein LDH-4 specific antibody levels were only 3-4% of those in serum (Kille and Goldberg, 1979). In contrast, in the mouse, immunisation with recombinant adenovirus or cholera toxin resulted in IgG titres in reproductive tract fluids that were more than 10% of those in serum (Gallichan and Rosenthal, 1995; Johansson *et al.* 1998). Taken together, these results suggest that

rabbits have a lower efficiency of IgG transfer from serum into the reproductive tract fluids than the mouse.

In rabbits, IgG staining and the number of IgG positive cells in reproductive tract tissues decreased after induction of ovulation (Chapter 3, section 3.3.8.2). But this did not affect the total or HA specific IgG concentrations in the luminal fluids. This is dramatically different from rodents where significant variations of IgG antibody levels in tissue or in the luminal fluids were demonstrated during oestrous cycles (Chapter 1 section 1.5.3). HA IgG antibody and total IgG titers were not significantly different between reproductive tract fluids obtained by flushing and micro-puncture. The flushing method resulted in antibody dilution with PBS whereas the micro-puncture method collected only the free fluid in the lumen. Since comparable antibody titers were obtained with these two methods, flushing samples must contain an IgG source that is not sampled by micropuncture. Epithelial mucus, which stains strongly for IgG (Chapter 3), is most likely to be the source of the additional IgG present in flushing samples. This suggests that, as for IgA, IgG is more concentrated at epithelial surfaces in rabbits rather than diffused into the fluids.

4.4.3 Myxoma virus is an effective vector for inducing a strong IgG response in serum and in the ovarian follicle

Although, as discussed above, the recombinant myxoma virus was not effective at inducing a strong antibody response in the reproductive tract, a consistent high-level IgG antibody response to HA was always induced in serum following any immunisation procedures. Therefore, it is assumed that good B memory cell, and probably T memory cell, responses to HA were generated. These results suggest that recombinant myxoma virus can be used as an effective vaccine vector to deliver rabbit antigens for disease control. A previous study in rabbits supporting this conclusion showed that recombinant myxoma virus expressing the rabbit hemorrhagic disease virus capsid protein could induce a protective immune response to this disease (Bertagnoli *et al.* 1996). It thus appears feasible that the recombinant virus could be used to deliver immunocontraceptive vaccines, but, if high antibody titers were needed, then the target antigen would have to be accessible to serum antibody.

In rabbits, previous studies showed that the IgG concentration to sperm antigen LDH-C4 in serum was comparable to that in ovarian follicular fluids (Symons and Herbert, 1971; Kille and Goldberg 1979). These data suggest that ovarian follicle antigens would be more accessible to serum antibody. To confirm this and extend it to viral vectored antigens, HA antibodies in ovarian follicular fluids were measured after MV-HA infection. In contrast to the mucosal compartments of the reproductive tract the antibody levels in ovarian follicular fluids were similar to those in serum, confirming that antibodies in serum freely transfer into the ovarian follicles. This result has important implications. It is hypothesized that antibodies to antigens in the zona pellucida (ZP), the layer covering the oocyte, play a major role in blocking fertilisation by coating the ZP of oocytes and preventing sperm attachment and penetration (Epifano and Dean, 1994; Aitken et al. 1996; Paterson et al. 2000). The free transfer of serum IgG antibody to the ovarian follicle implies that if a strong IgG antibody response to a ZP antigen was induced in serum the antibody would enter the follicle and bind to the ZP. This might either damage the oocyte clinically or prevent sperm binding to the ovulated oocytes.

The recombinant myxoma virus has the ability to deliver an antigen and induce a strong and lasting antibody response in serum. Therefore, it could be used as a vector to deliver ZP antigen for immunocontraception. During the course of this study, antibody levels in serum of rabbits immunised with zona pellucida protein B (ZPB) correlated with the extent of the resultant infertility (Kerr, et al. 1999). Kerr et al (1999) also showed that immunisation of female rabbits with a recombinant myxoma virus expressing rabbit ZPB induced a low antibody response to ZPB with 25% infertility in infected rabbits. In addition, it was shown that female mice immunized with a recombinant ectromelia virus expressing mouse ZP3 became infertile and antibody to ZP3 played an important role in this (Jackson et al. 1998). These results demonstrate that ZP antigens can induce infertility in mice when delivered by a recombinant virus but that recombinant myxoma virus was not an effective system in rabbits compared with protein immunization. Other ovarian antigens such as membrane proteins from the granulosa or cumulus cells could also be considered for developing viral vectored immunocontraception.

4.4.4 A summary

In summary, the study has shown that the recombinant myxoma virus is inefficient at inducing an antibody response in the female rabbit reproductive tract. The high-level IgG antibody in circulation did not transfer into the reproductive tract fluids at high titer. Therefore, this vector is not suitable for inducing an immune response in the mucosal part of the reproductive tract. However, the recombinant virus always induces a strong systemic antibody response to the vectored antigen and this is readily transferred to the ovarian follicle. This provides an opportunity to use recombinant MV as a vector to deliver an immunocontraceptive vaccine targeted at the ovary.

CHAPTER 5: IMMUNOLOGICAL AND HISTOLOGICAL RESPONSES IN OVARY TO INFECTIONS OF A RECOMBINANT MYXOMA VIRUS EXPRESSING RABBIT ZONA PELLUCIDA ANTIGEN B (ZPB)

5.1 Introduction

In Chapter 4, it was shown that the recombinant myxoma virus could induce a strong serum antibody response to a vectored foreign antigen but only a weak antibody response in the reproductive tract. However, high levels of antibodies were achieved in the ovarian follicles suggesting that if recombinant myxoma virus is used to deliver an immunocontraceptive vaccine, the targeted antigen needs to be accessible to serum antibodies. Ovarian follicular antigens meet this need better than antigens that are only present in the lumen of the reproductive tract and therefore should also be considered as targets for immunocontraception.

Many studies have already shown that infertility can be achieved by immunisation with zona pellucida (ZP) antigens (Chapter 1 section 1.6.1). However, very little is known about the immune response in the ovary and the immunological mechanism of the infertility induced by immunization with such antigens, particularly following their presentation by a recombinant virus.

The zona pellucida (ZP) is an extracellular matrix surrounding the oocytes and plays a role in oocyte development and fertilisation where it provides a receptor for sperm binding (Epifano and Dean, 1994). Like most species, the rabbit ZP consists of three

major glycoproteins ZPA, ZPB, and ZPC with predicted molecular weights of 75, 55, and 45 Kd respectively (Dunbar *et al.* 1994). The rabbit ZPB was shown to be the homologue of porcine ZP3α that is considered to be the primary receptor for sperm binding in the pig (Dunbar *et al.* 1994). It therefore may have the potential to be an immunocontraceptive antigen for rabbits. Antibodies to this antigen could act by one of two mechanisms, by destruction of ovarian follicles or by blocking sperm binding to the oocyte and hence blocking fertilization.

Immunisation with rabbit native ZP protein or recombinant ZPB expressed in *E.coli* did not induce either antibody to ZP or ZPB or infertility in rabbits (Chapter 1 section 1.6.1). The immunogenicity of rabbit recombinant ZPB was enhanced by expression in eukaryotic cells (Prasad *et al.* 1995) and during this PhD course, Kerr et al. (1999) showed that immunisation of female rabbits with rabbit ZPB that had been expressed in a rabbit cell line resulted in 75% infertility. However, although rabbit ZPB delivered by a recombinant myxoma virus (MV-ZPB) could induce an antibody response in female rabbits, the resulting infertility rate was only 25% and was not sustained (Kerr *et al.* 1999). These data suggested that glycosylation of ZPB in eukaryotic cells was an important determinant affecting the immune response needed for immunocontraception and that using recombinant myxoma virus to deliver the self-antigen, recombinant rabbit ZPB, is a prospective method for achieving immunocontraception, if infertility rates could be increased. However, the immunological and histological responses in the ovary during the course of the MV-ZPB infection were not investigated by Kerr et al. (1999). This is necessary for understanding the mechanism of infertility and to provide a basis for further development of an effective viral vectored immunocontraceptive vaccine.

Immunisation with ZP antigens often causes ovarian pathology, follicle disruption and ovarian dysfunction (Chapter 1 section 1.6.2.2) which, in rabbits and other species, were shown to be closely linked to the infertility rate (rabbits: Wood *et al.* 1981; Skinner *et al.* 1984; Jones *et al.* 1992; Kerr *et al.* 1999; mice: Jackson *et al.* 1998; marmoset monkey: Aitken *et al.* 1996; bonnet monkeys: Bamezai *et al.* 1986, and hamster: Hasegawa *et al.* 1992). In the rabbit, ZP antibody binding to oocytes was suggested to play a major role in follicle disruption and ovarian dysfunction (Skinner *et al.* 1984), although the pathological mechanism of these effects remains unclear. In

addition to the potential effects of antibody, ZP specific cytotoxic or regulatory T cells can play a role in ovarian pathogenesis in mice (Tung and Teuscher, 1995; Garza, Lou, and Tung, 1998; Tung *et al.* 2001). It is unknown in rabbits whether a T cell response contributes to the ovarian pathology following ZP immunisation.

In this chapter, a recombinant myxoma virus expressing rabbit ZPB (MV-ZPB, Chapter 2 section 2.1.1.1) was employed to investigate the immunological and pathological responses in the ovary following infection of rabbits with the virus.

5.2 Experimental design

Experiment 1. Time course studies of MV-ZPB infection. Twelve female rabbits were intra-dermally immunised with 1 x 10^3 pfu of MV-ZPB (Chapter 2 section 2.2.2.1). Four rabbits were killed at day 5, four at day 15 and four at day 30 post-infection (termed as MV-ZPB D5, D15 and D30 groups in following text). Blood was collected for preparing serum for antibody measurement and ovaries were collected for preparation of frozen and paraffin embedded sections (Chapter 2 section 2.2.3.3, 2.2.3.4). As an infected control, another 8 female rabbits infected with 1 x 10^6 pfu MV-HA (Chapter 2 section 2.2.2.1) were killed at day 15 and day 30 post-infection (4 rabbits at each time point, termed as MV-HA D15 and D30 groups) and blood and ovaries were collected as above. Another group of four normal female rabbits was killed as an uninfected control group. Blood and ovaries were collected for the same analyses.

Experiment 2. The relationship between follicle reduction and infertility. To compare the immunological and histological changes in ovaries from rabbits infected with MV-ZPB alone with rabbits infected with MV-ZPB and further boosted with recombinant ZPB protein (Kerr *et al.* 1999), four normal female rabbits were intradermally infected with 1x 10^3 MV-ZPB and boosted twice with purified recombinant ZPB protein (Chapter 2 section 2.2.2.1) at day 30 and day 44 post-infection. Blood and ovaries were collected for analysis when the animals were killed at 58 days post-infection.

Experiment 3. Comparison of expanded interstitial cells and granulosa lutein cells in the ovary. After MV-ZPB infection, numerous large pale-staining cells appeared in the

ovary which resembled expanded interstitial or granulosa lutein cells. To compare these cells with granulosa lutein cells in the ovulated ovary, two female rabbits were injected with hCG to induce ovulation (Chapter 2 section 2.2.2.2) and another two were mated to induce ovulation. The four rabbits were killed 5 days later and ovaries were collected for histological analysis.

5.3 Results

5.3.1 Serum antibody response to ZPB following infection with MV-ZPB

Six days after inoculation with MV-ZPB, the skin at the inoculation site developed a typical primary lesion. Around 15 days, all rabbits developed myxomatous lesions on their ears, eyelids or noses. The rabbits started to recover about day 20 and by day 30 post-infection the rabbits had recovered from myxomatosis and scars had replaced the lesions.

IgG antibody titers to rabbit ZPB in serum were measured using ELISA (Chapter 2 section 2.3.1). ZPB IgG antibody was first detected in two of the four rabbits at 5 days post-infection, peaked at day 15 and had dropped at 30 days post-infection (Table 5-1). No IgG antibody to ZPB was detected in pre-immune serum of those rabbits or in serum from uninfected or MV-HA infected rabbits.

Table 5- 1. IgG antibody to rabbit ZPB in serum of rabbits infected with MV-ZPB

Day 5		Day 15		Day 30	
Rabbit No.	Titer (Log_{10})	Rabbit No.	Titer (Log_{10})	Rabbit No.	Titer (Log_{10})
515	1.7	511	2.9	505	2.0
520	1.4	517	3.8	461	2.0
522	0	488	3.5	521	2.6
479	0	509	2.6	503	2.6
Mean ± S.D	0.78 ± 0.9	3.2 ± 0.5		2.3 ± 0.3	

5.3.2 Antibody localisation in the ovary following MV-ZPB infection

To determine whether the serum ZPB antibodies were also bound to oocytes, frozen sections of ovaries from all groups were stained for rabbit IgG, IgM and IgA with goat anti-rabbit IgG and IgA sera and anti-rabbit Ig μ-chain monoclonal antibody (Chapter 2 Table 2-1 and 2). Normal goat serum or MAF were used as negative controls for the appropriate sections.

5.3.2.1 IgG staining in the ovary

At day 5 post-infection (MV-ZPB D5 group) no IgG staining was found on the ZP in follicles at any developmental stage (Figure 5-1). Similarly, IgG was not detected on the ZP or on any cells in the negative controls treated with normal goat serum instead of the primary antibody (Figure 5-1). However, at day 15 post-infection (MV-ZPB D15 group) strong IgG staining was seen on the ZP of oocytes in most secondary and tertiary follicles (Figure 5-2); this staining had increased at 30 days (Figure 5-3). Phase contrast images confirmed that the whole ZP and only the ZP of the oocytes stained strongly for IgG (Figure 5-2, 5-3). IgG staining was not usually found on the ZP in primary or primordial follicles in these groups. Very weak IgG staining was occasionally seen on the ZP in ovaries from MV-HA infected rabbits and uninfected controls, suggesting that a small amount of IgG binds non-specifically to the ZP (Figure 5-4). These results suggested that the IgG bound to ZP in the ovaries was specific IgG antibody to ZPB produced by immunisation with MV-ZPB.

5.3.2.2 IgM staining in the ovary

IgM bound weakly to the ZP in only a few ovarian follicles from any of the infected groups (Figure 5-5) though it has been shown to enter ovarian follicular fluids freely (Chapter 4 section 4.3.9). This lack of binding may reflect a low level of IgM antibody in serum although this was not measured in these experiments.

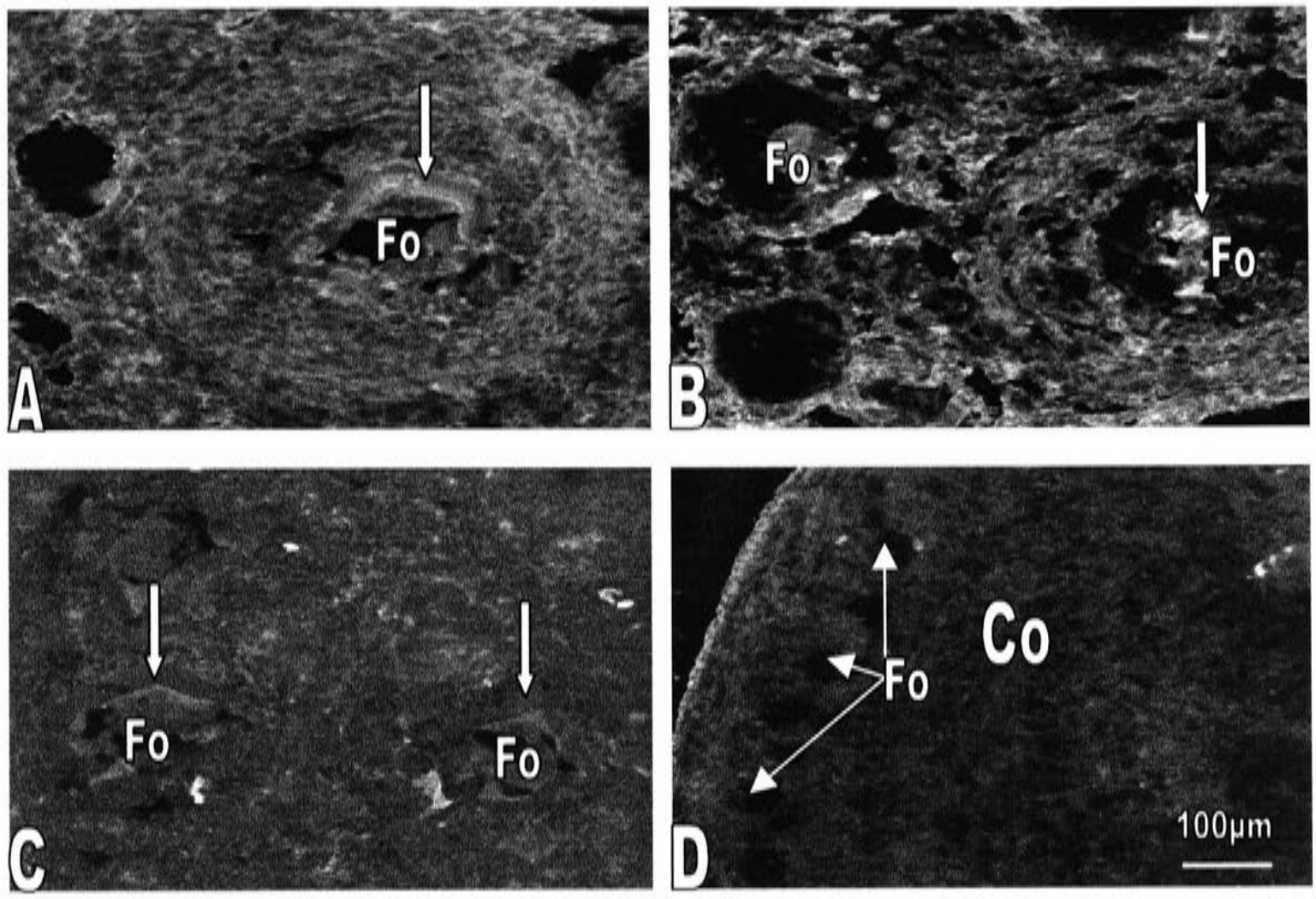


Figure 5- 1. IgG staining in ovaries of rabbits at day 5 post-infection with MV-ZPB and negative controls. Images A and B are from two rabbits in the MV-ZPB D5 group and show no IgG staining on the ZP of the oocytes (A, arrow) or in the follicles (B, Fo) though weak background staining could be seen on some granulosa or cumulus cells in one follicle (B, arrow). Images C and D show the negative controls treated with normal goat serum instead of the primary antibody. No IgG staining could be seen on the ZP of oocytes (C, arrows) or in the follicles (D). Fo: follicles. Co: cortex. All images are at the same magnification.

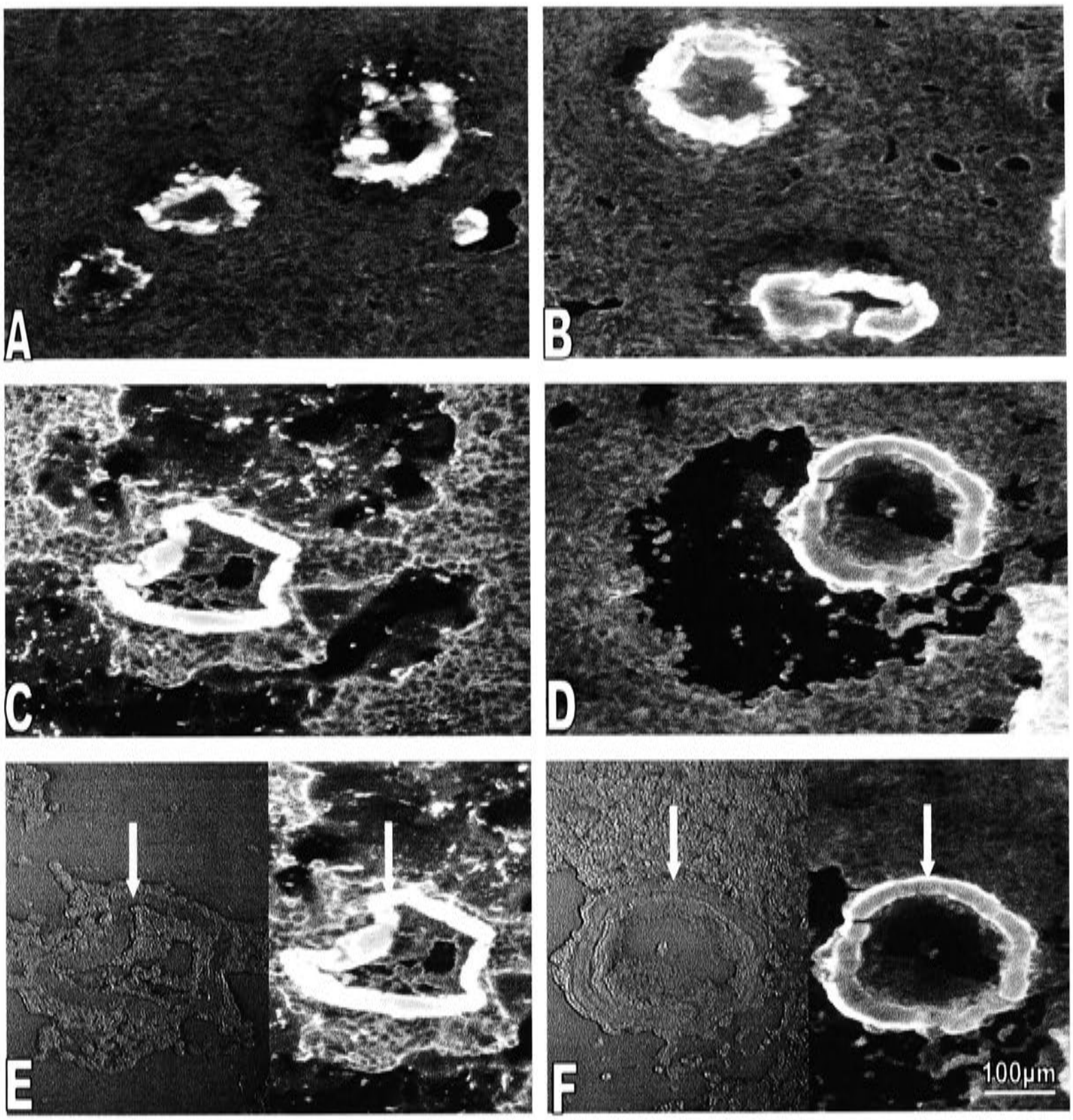


Figure 5- 2. IgG localisation in ovaries of rabbits at 15 days post-infection with MV-ZPB. The images A, B, C and D (from 3 different infected rabbits) show that strong IgG staining is localised on the ZP of oocytes in secondary (A and B) and tertiary (C and D) follicles. E and F show phase contrast images of the follicle structure and ZP location (left panel of E and F, arrows) and corresponding IgG fluorescent images (right panel, arrows), confirming strong IgG staining localised on ZP. All images are at the same magnification.

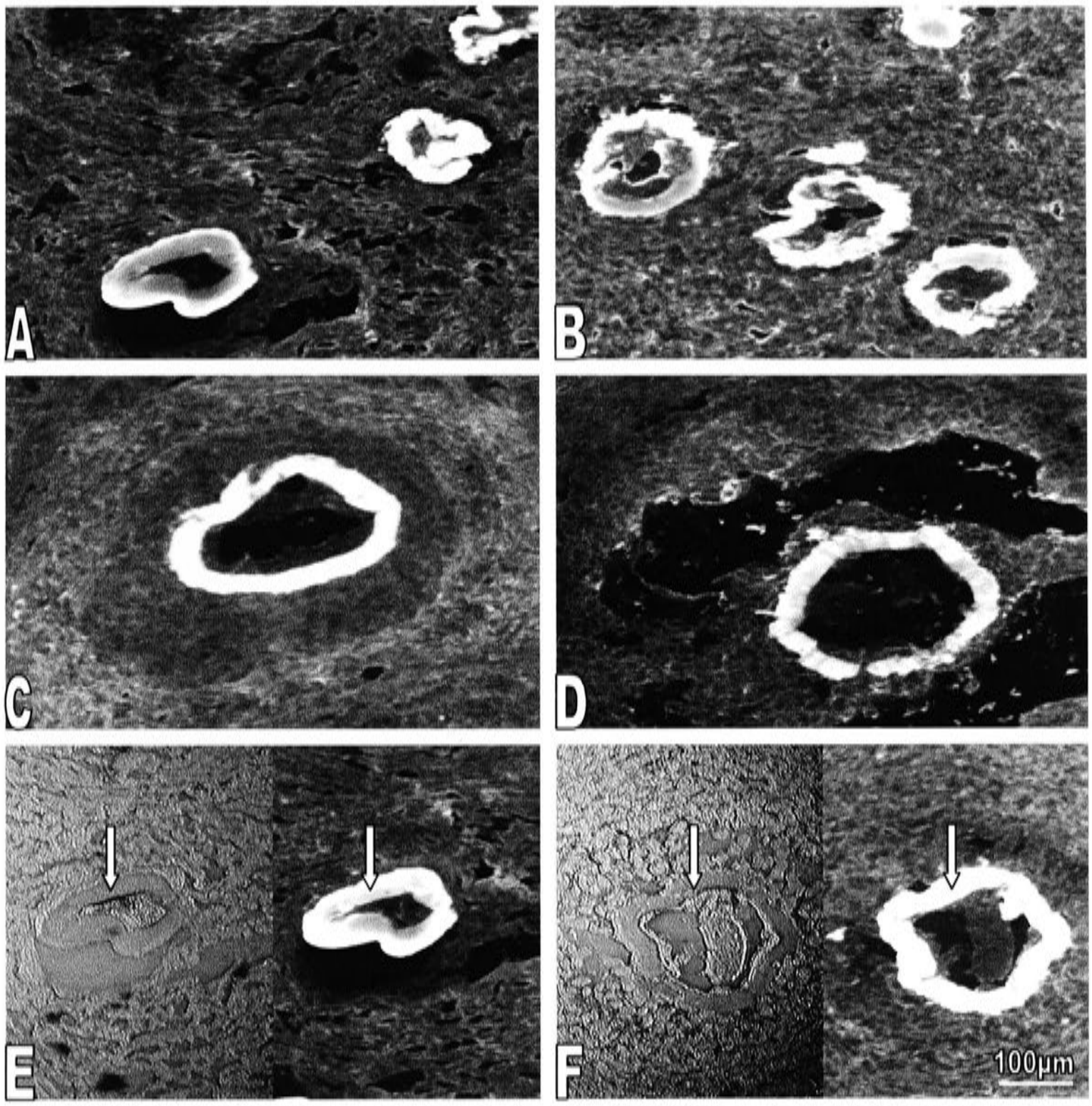


Figure 5- 3. IgG localisation in ovaries of rabbits at 30 days post-infection with MV-ZPB. A, B, C, and D are from 3 different rabbits at 30 days post-infection and show strong IgG staining on the ZP of oocytes in secondary follicles of different sizes (A, B, and C) and tertiary follicles (D). E and F are phase contrast images showing the morphology of the follicles (left side, ZP is labelled with arrow) and corresponding fluorescent images (right side, arrow). All images are at the same magnification.

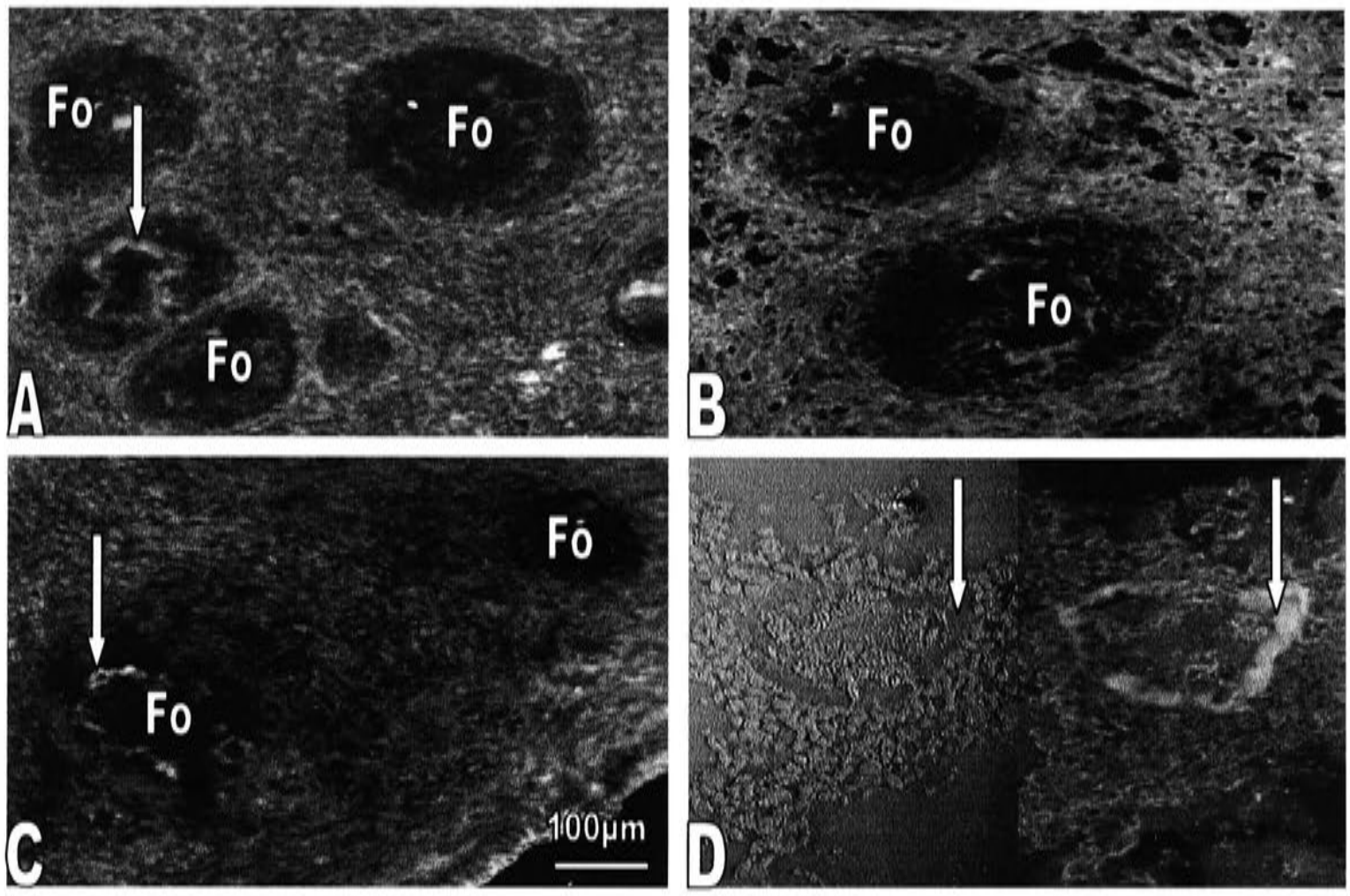


Figure 5- 4. IgG staining in ovaries of rabbits infected with MV-HA or from uninfected controls. Images A and B are ovarian sections from rabbits infected with MV-HA at 15 and 30 days after infection. Image C is an ovary from an uninfected control. Weak IgG staining was seen in some follicles (arrow A & C) but not in most. Image D is also from an uninfected control and consists of two parts: the left part is a phase contrast image showing the structure of the ZP (arrow), the right part is the corresponding fluorescent image which shows very weak IgG staining on the ZP in a tertiary follicle (arrow). Fo: follicle. All images are at the same magnification.

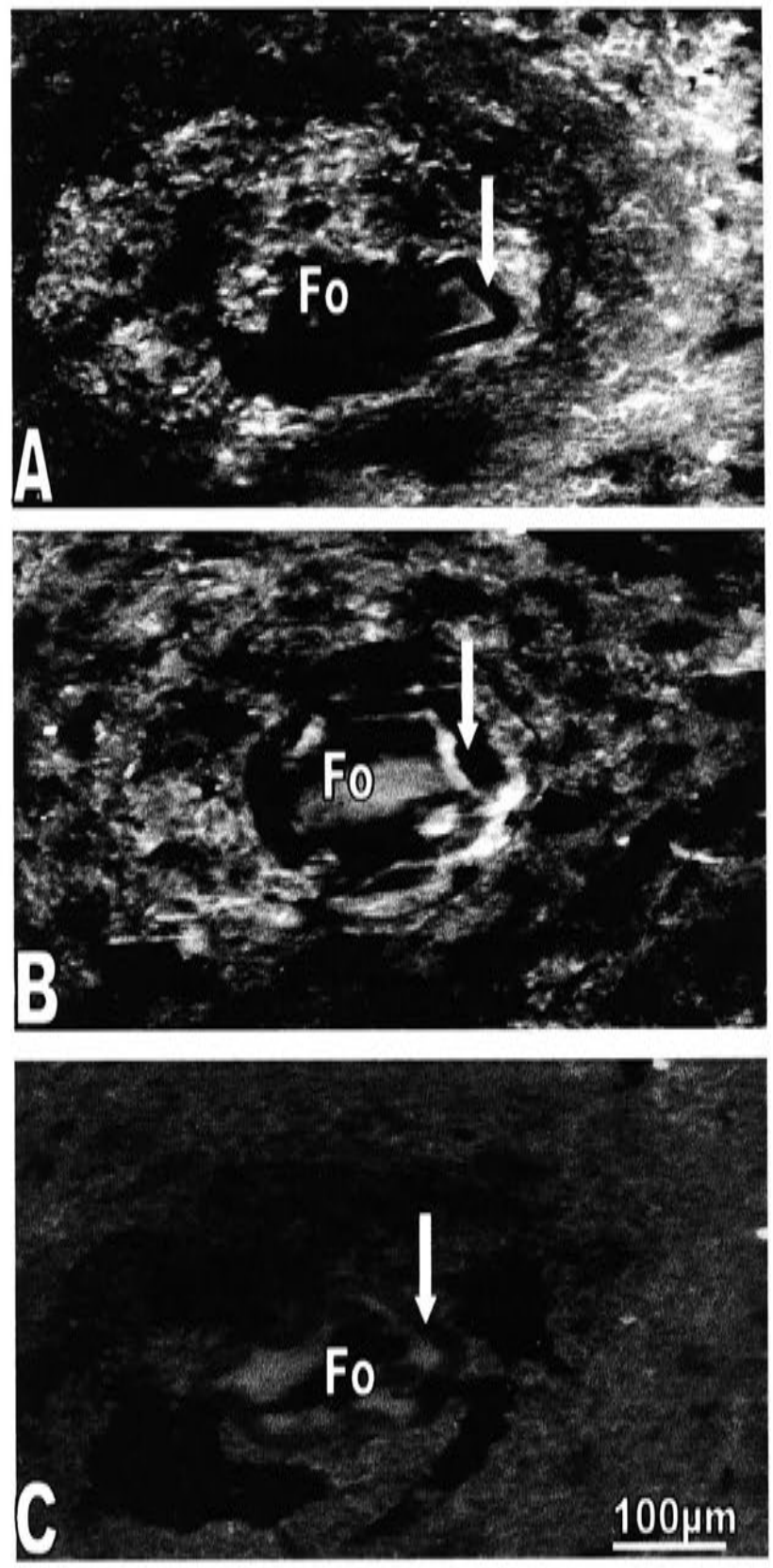


Figure 5- 5. IgM staining in the ovary of rabbits infected with MV-ZPB. IgM staining in ovaries from rabbits at day 15 (A) and 30 (B) post-infection with MV-ZPB. No IgM staining was found on ZP of oocytes (A, B arrow) Ig µ-chain positive cells were found around the follicles especially in A (A and B, Fo), and granulosa or cumulus cells were also weakly positive (A). Image C shows a negative control of ovary from a MV-ZPB infected rabbit treated with MAF. Fo: follicle. All images are at the same magnification.

The presence and location of B cells in the ovaries was also examined after staining with anti-rabbit Ig μ-chain monoclonal antibody. Whereas B cells were rarely seen in ovaries from uninfected controls, B cells were found surrounding some primary and secondary follicles in 3 of the 4 rabbits in the MV-ZPB D5 group (Figure 5-6). In the MV-ZPB D15 group, follicles of all stages were positive for B cells (Figure 5-6) but far fewer B cells were present around the follicles in the MV-ZPB D30 group. In MV-HA infected rabbits, only a few B cells were present in the cortex or medulla or sometimes around the follicles of any ovaries at day 15 and day 30. This distribution was very similar to the MV-ZPB D30 group. No positive cells were seen on the sections treated with MAF instead of the antibody. The peak in B cell numbers at day 15 suggests that an inflammatory reaction may have occurred in the ovaries of MV-ZPB infected rabbits between 5 and 15 days post-infection.

5.3.2.3 IgA staining in the ovary

No IgA binding was observed in any ovarian sections.

5.3.3 Specificity of serum IgG antibody

5.3.3.1 Serum antibody binding to the zona pellucida in ovarian sections

To confirm the specificity of serum ZP-IgG antibody, frozen sections of ovaries from the MV-ZPB D5 group, which have previously been shown not to have antibody binding to the oocytes (Section 5.3.2.1), were incubated with 1:100 diluted positive sera (equal volumes of sera pooled from two rabbits from the MV-ZPB D15 and D30 groups). Binding of IgG was assessed by incubation with anti-rabbit IgG conjugated with FITC (Chapter 2 Table 2-3). Most zona pellucida of oocytes in secondary and tertiary follicles were strongly positive (Figure 5-7), which confirmed that the IgG antibody in the serum could specifically bind to the ZP *in vitro*. Pooled sera from MV-HA infected rabbits were used as controls; no IgG staining was found on any ZP in sections treated with this serum.

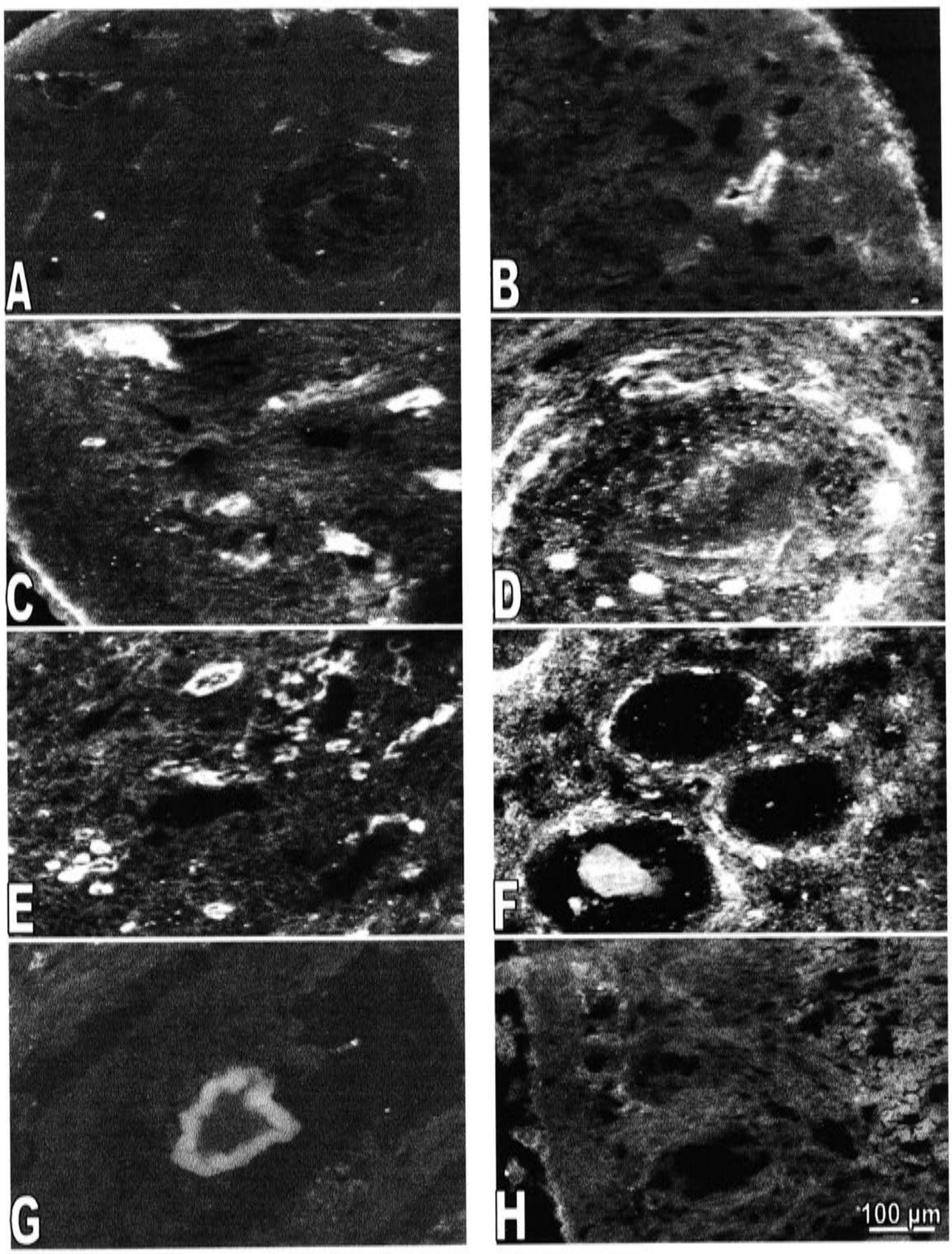


Figure 5- 6. Ig µ-chain positive B cells in ovaries of rabbits infected with MV-ZPB. Very few Ig µ-chain positive cells were present in ovaries from uninfected (A) or MV-HA infected (B) controls. More positive cells were found in the MV-ZPB infected rabbits at day 5 (C and D) and day 15 (E and F). Most of these cells were associated with follicles. At day 30 an occasional ZP was weakly positive for IgM (G) but few B cells were present in the ovary (H). All images are at the same magnification.

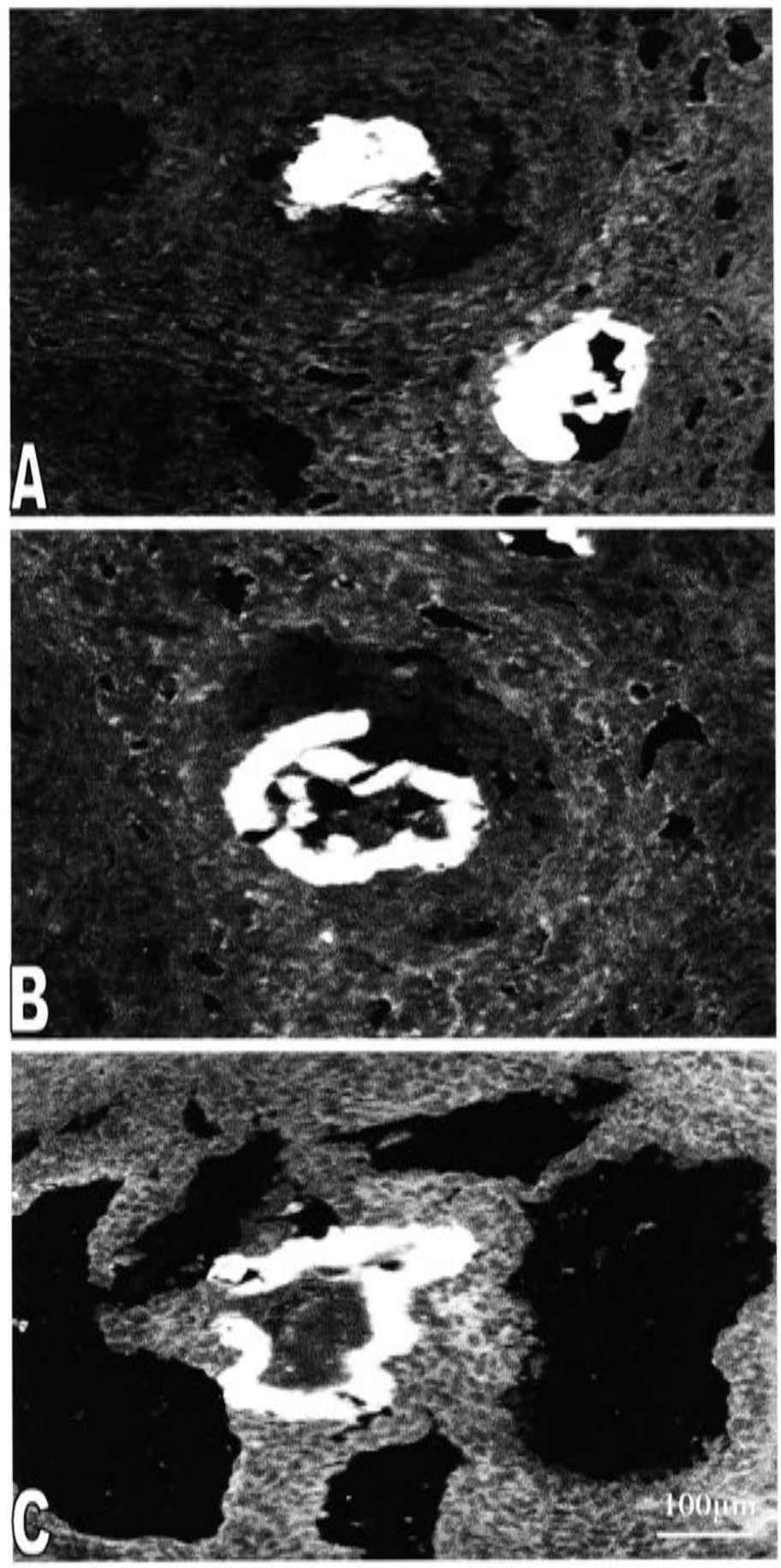


Figure 5- 7. Specificity of serum IgG antibody from rabbits infected with MV-ZPB. The zona pellucida in secondary (A and B) and tertiary (C) follicles of rabbits at 5 days post-infection, which were negative for IgG (Figure 5-3), were stained using positive serum from rabbits at 15 or 30 days post-infection as primary antibody and detected by FITC antibody conjugate. All images are at the same magnification.

5.3.4 T cell response in the ovary after MV-ZPB infection

To examine the cell-mediated immune response in the ovary after MV-ZPB infection, the presence of rabbit T cells was assessed in frozen sections of the ovary by immunofluorescence (Chapter 2 section 2.4.1) using monoclonal antibodies to rabbit KEN-5 and CD43 (Chapter 2 Table 2-1). Since there were differences in the staining patterns of these two monoclonal antibodies (Chapter 3 section 3.4.1) both were used. MAF was used as the negative control.

5.3.4.1 KEN-5 positive cells in the ovary

KEN-5+ cell infiltrations were found in only one of four rabbits in the MV-ZPB D5 group and one of four in the MV-ZPB D15 group. The KEN-5+ cells were present in the cortex of the ovary, mostly located closely around secondary or tertiary follicles (Figure 5-8). Very few KEN-5+ cells were found in the ovaries of the other three rabbits in these two groups. In the MV-ZPB D30 group and the MV-HA group, only a few positive cells were present. These ovaries stained similarly to those of the negative rabbits in the day 15 group. Ovaries from the uninfected controls had some KEN-5+ cells but far fewer than in the positive rabbits from the MV-ZPB day 5 and day 15 groups; the positive cells were not located round the follicles. No positive cells were seen in the negative control stained with MAF.

5.3.4.2 CD43 positive cells in the ovary

More CD43 positive cells than KEN-5+ cells were present in ovaries from all infected and uninfected groups. Large increases in CD43+ cell numbers were observed in the two rabbits from the MV-ZPB D5 and D15 groups that had also shown a substantial increase in KEN-5+ cell number; most CD43+ cells were located closely around secondary and tertiary follicles (Figure 5-9). In two adjacent ovarian sections from these rabbits labeled with CD43 and KEN-5, most CD43+ cells were also KEN-5+ cells though CD43 staining was stronger than KEN-5 (Figure 5-9). The numbers and distribution of CD43+ cells in the other 3 rabbits in the MV-ZPB day 5 and day 15 groups, and in all rabbits in the MV-ZPB day 30 group, were similar to the uninfected controls. CD43+ cell numbers and locations in MV-HA infected rabbits did not appear

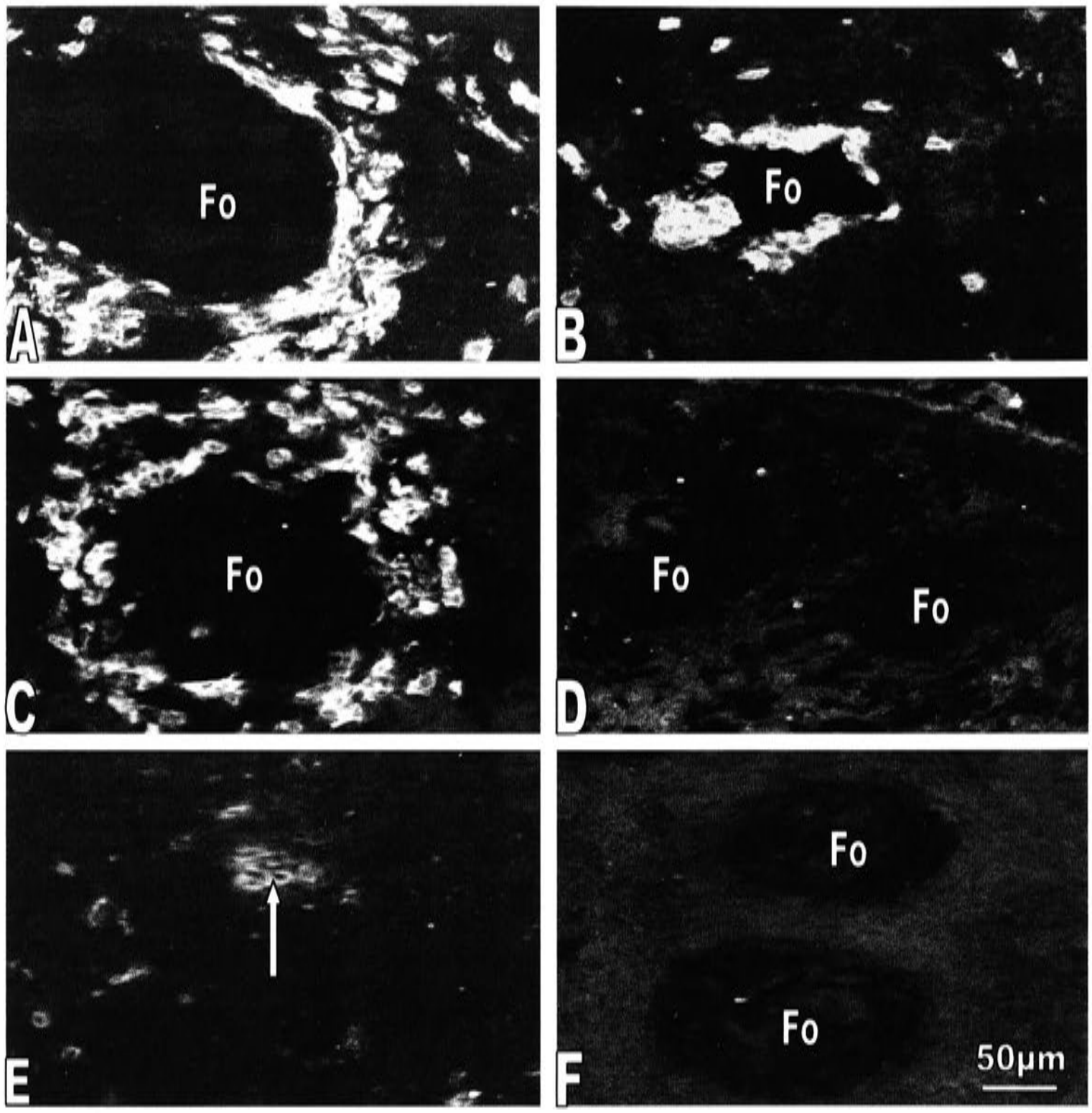


Figure 5- 8. KEN-5+ T cell response in ovaries of some MV-ZPB infected rabbits. Many KEN-5+ T cells were present around the follicles (Fo) in one of 4 MV-ZPB infected rabbits at 5 days post-infection (A, B) and one of 4 rabbits at 15 days post-infection (C). In contrast, no (D) or very few (E) positive cells were present in ovaries from other infected rabbits including the day 30 group (D) and uninfected controls (E). F is ovary from a MV-ZPB infected rabbit at 5 days post-infection treated with MAF. All images are at the same magnification.

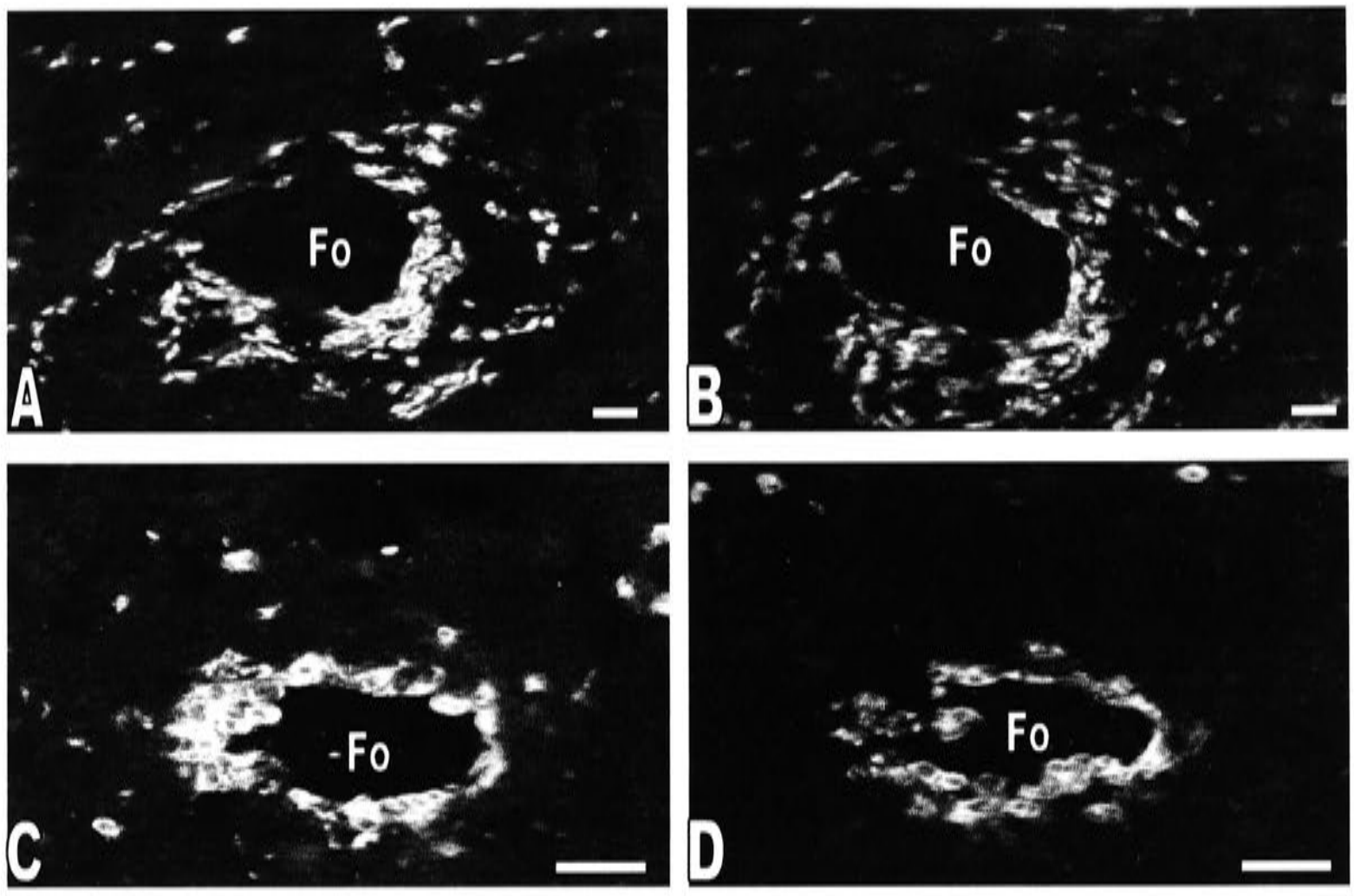


Figure 5- 9. Comparison of CD43+ and KEN-5+ T cells in ovaries from MV-ZPB infected rabbits. The images show CD43+ (A and C) and KEN-5+ (B and D) T cell labelling in two serial ovarian sections (A and B are from the positive rabbit in the day 5 group and C and D are from the positive rabbit in the day 15 group of MV-ZPB infection). Most of the CD43+ and KEN-5+ cells were present round the follicles (Fo). The scale bars represent 50 micro-meters.

different to those from uninfected control rabbits. However, in 2 of 4 day 15 rabbits, estimated numbers of CD43+ staining cells were intermediate between those in uninfected control sections and in the ZPB sections that were positive for T cell staining. Overall, it is difficult to draw firm conclusions about the role of T cells in ovarian pathology following immunization with MV-ZPB.

5.3.5 Morphological and histological changes in the ovary after MV-ZPB infection

5.3.5.1 Ovarian weight and pro-ovulatory follicle losses

Obvious changes in ovarian appearance were noticed at the time that the ovaries were collected for analysis. Ovaries from infected rabbits (both MV-ZPB and MV-HA) were flattened and smaller than those from uninfected controls (Table 5-2). Most ovaries in the MV-ZPB D15 and D30 groups had fewer pre-ovulatory follicles protruding at surfaces than uninfected controls. There were no differences in ovarian weight between MV-ZPB and MV-HA infected rabbits at either day 15 or day 30. Although the mean weight of the left ovaries in the MV-ZPB D5 group was lower ($P<0.05$) than that of uninfected controls the same was not true for the right ovary ($P=0.067$). Rabbits were randomly selected by animal house staff and there was no correlation between body weight and ovarian weight in any group.

Table 5- 2. Fresh ovarian weight of rabbits infected with MV-ZPB or MV-HA

Groups	Left ovary (gram)	Right ovary (gram)
Controls	0.32 ± 0.04	0.33 ± 0.04
MV-ZPB D5	0.22 ± 0.05*	0.25 ± 0.06
MV-ZPB D15	0.15 ± 0.05**	0.13 ± 0.06**
MV-HA D15	0.16 ± 0.07**	0.16 ± 0.06**
MV-ZPB D30	0.19 ± 0.05**	0.19 ± 0.07*
MV-HA D30	0.13 ± 0.03**	0.14 ± 0.02**

Notes: the values are mean weight ±S.D of 4 rabbits in each group. Statistical analysis: *: $P<0.05$ against controls; **: $P<0.01$ against controls.

The right ovaries from each rabbit were collected for histological examination (Chapter 2 section 2.2.3.4). Four to six longitudinal sections at 6-8 micrometer thickness were cut from each ovary. Fewer pre-ovulatory follicles were seen in sections from MV-ZPB infected ovaries than those of uninfected and MV-HA infected controls (Figure 5-10). To quantify this, the pre-ovulatory follicles (>0.5mm in diameter) in a section from each rabbit were counted and the average follicle number of each group is shown in Table 5-3. Compared with the uninfected controls and MV-HA D30 groups, MV-ZPB D30 group had significantly fewer follicles ($P<0.05$ for both, Table 5-3).

Table 5- 3. The number of pre-ovulatory follicles (>0.5 mm in diameter) in ovarian sections

Groups	Number of pre-ovulatory follicles (>0.5 mm in diameter) per section
Control	6.5 ± 1.7
MV-ZPB D5	5.0 ± 2.2
MV-ZPB D15	4.3 ± 1.9
MV-HA D15	4.3 ± 1.3
MV-ZPB D30	2.3 ± 1.5*
MV-HA D30	5.8 ± 1.3

Notes: the values are mean ±S.D of 4 rabbits for each group. *: $P<0.05$ with uninfected and MV-HA infected control groups.

5.3.5.2 Follicle loss and degeneration

A decrease in follicle numbers was observed in ovaries from most MV-ZPB infected rabbits compared with controls. To quantify this, the numbers of primary, secondary and tertiary follicles in these sections were counted. Compared with uninfected controls, MV-ZPB infected rabbits suffered a significant ($P<0.05$) loss of both secondary and tertiary follicles at 30 days post-infection but not at 15 and 5 days post-infection (Table 5-4, Figure 5-11). The number of tertiary, but not secondary follicles was also significantly lower than that in the corresponding MV-HA infected group ($P<0.05$, Table 5-4). The number of primary follicles was not significantly affected in any MV-

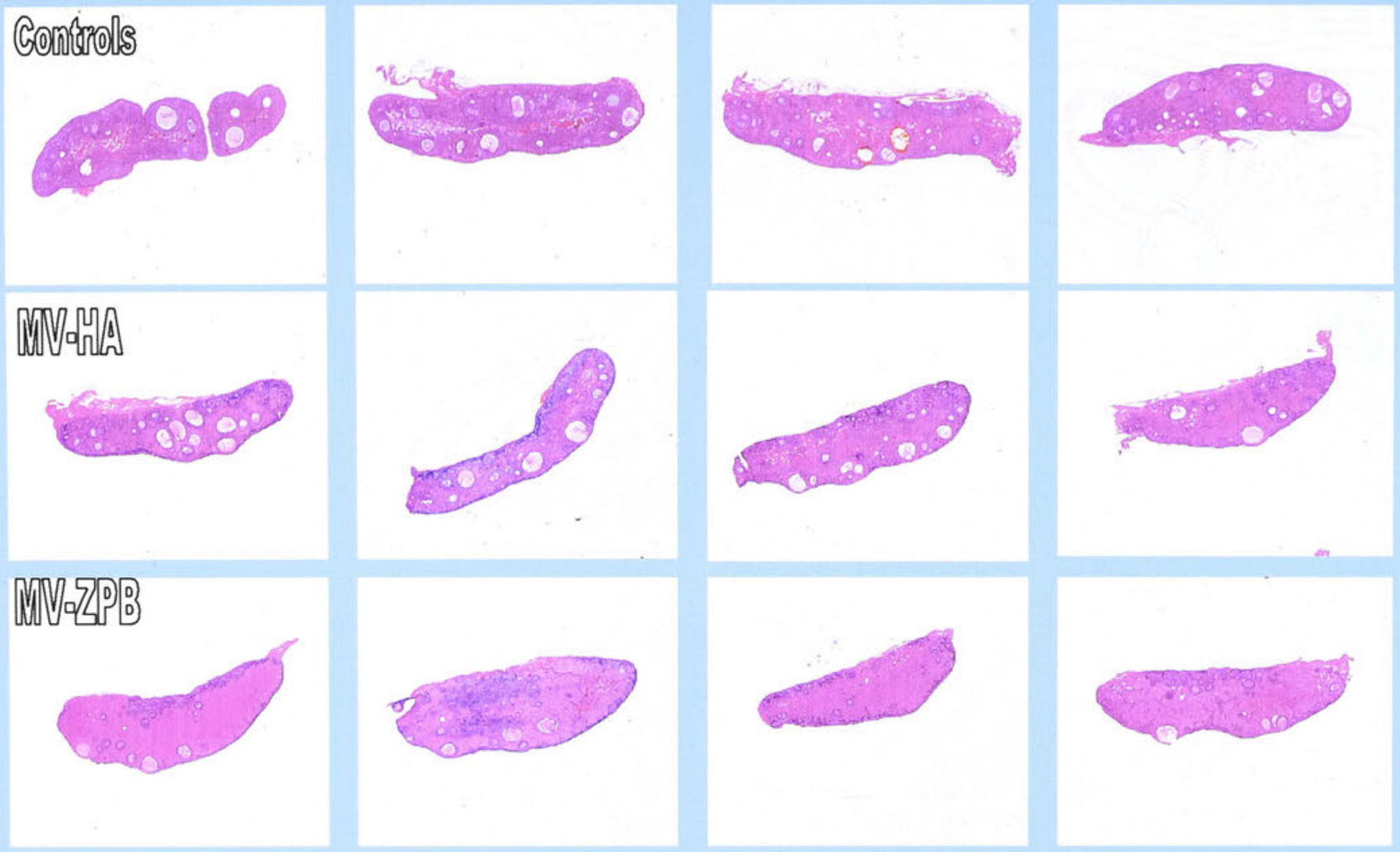
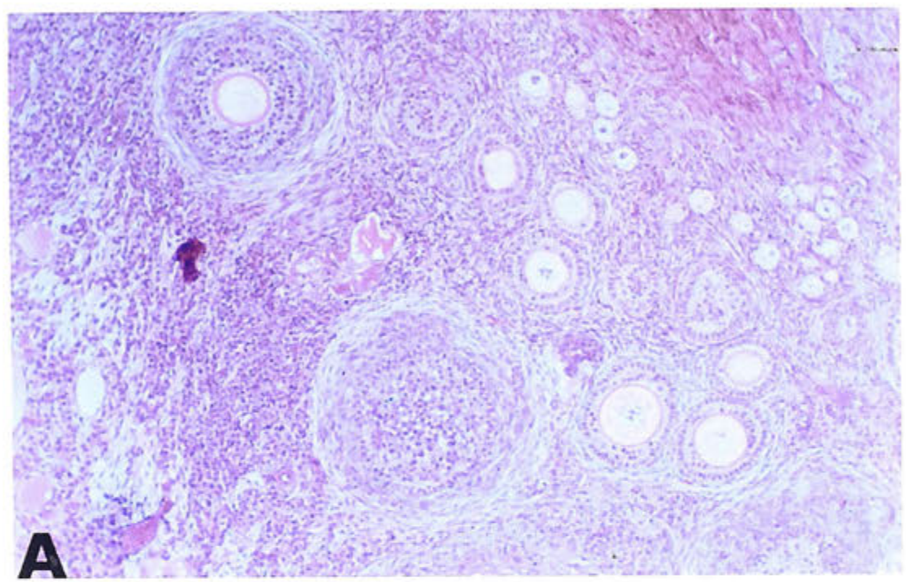


Figure 5- 10. The loss of pre-ovulatory follicles in MV-ZPB infected ovaries. Compared with the ovarian sections from uninfected (Control) and MV-HA infected (MV-HA) controls, the numbers of pre-ovulatory follicles in the 4 ovarian sections of MV-ZPB infected rabbits (MV-ZPB, at 30 days post-infection) were markedly reduced. Each of those sections is representative of 4-6 ovarian sections obtained from one rabbit. All images are 2x magnification.

Figure notes are on the other side

Figure 5- 11. Reduction in follicle numbers in ovaries from MV-ZPB infected rabbits (100x).

A is from an uninfected control rabbit; B is from a rabbit at day 5 post-infection with MV-ZPB; C is from a rabbit at day 30 post-infection with MV-ZPB; D is from a rabbit at 30 days post infection with MV-HA.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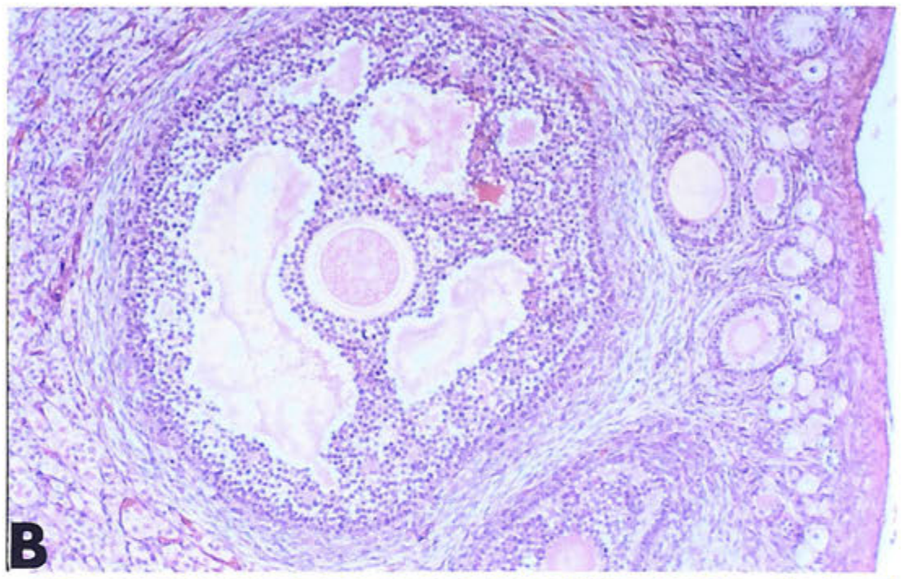
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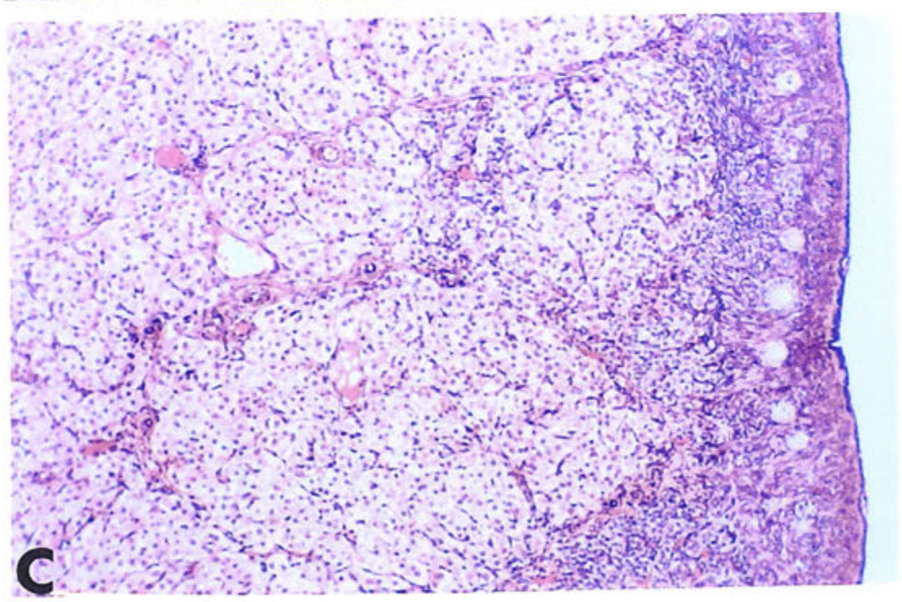
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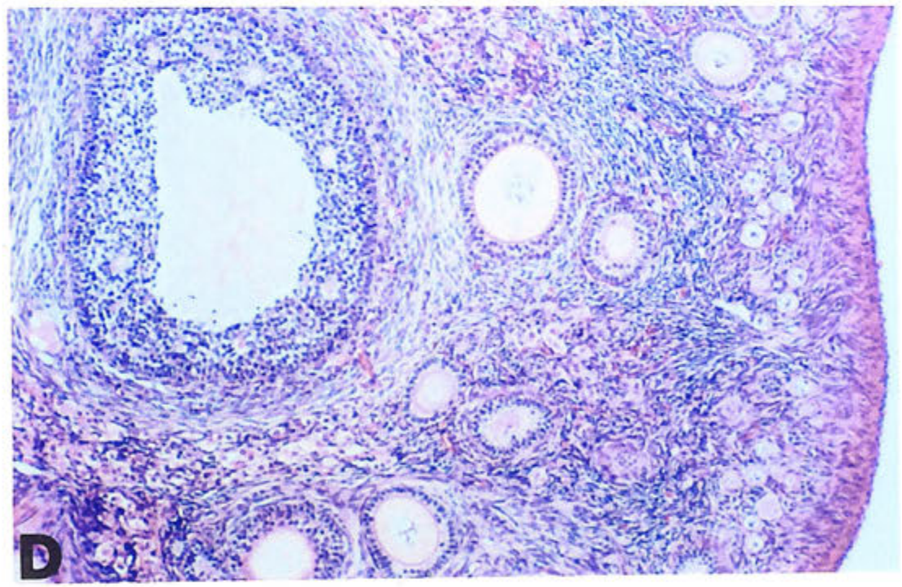

D

ZPB infected group compared to the uninfected and MV-HA infected controls (Table 5-4). The numbers of tertiary, secondary and primary follicles in MV-HA infected rabbits were not significantly different from uninfected controls.

Table 5- 4. The numbers of follicles at different stages of development

Groups	Primary follicles	Secondary follicles	Tertiary follicles
Controls	32.5 ± 5.8	28.8 ± 6.4	14.8 ± 1.7
MV-ZPB D5	33.8 ± 7.3	25.8 ± 3.3	15.3 ± 3.3
MV-ZPB D15	39.5 ± 20.7	23.8 ± 15.1	14.5 ± 2.4
MV-HA D15	32.3 ± 18.4	25.8 ± 11.3	12.0 ± 3.6
MV-ZPB D30	29.5 ± 9.6	14.5 ± 4.9*	8.5 ± 4.1*
MV-HA D30	29.8 ± 13.9	20.8 ± 4.6	16.3 ± 3.9

Notes: the values are mean ± S.D of 4 rabbits for each group. *: $P<0.05$ against uninfected controls for secondary follicles and against uninfected and MV-HA infected controls for tertiary follicles. The primary follicles were classified as follicles with one-layer of granulosa cells. Secondary follicles were classified as follicles with two or more layers of granulosa cells but without an antrum. Tertiary follicles were defined as follicles with multiple layers of granulosa cells and an antrum. One section from the same ovarian location for each rabbit was counted.

Although the number of secondary follicles in the MV-ZPB D30 group was not statistically different from the MV-HA D30 group, there was an obvious difference in follicle distribution between these two groups. No secondary follicles were seen in large areas of the cortex in ovaries from the MV-ZPB D30 group (4 of 4 rabbits) and these areas were infiltrated with many large pale-staining cells (Figure 5-11). These cells resembled the expanded interstitial cells described by Hammond and Marshall (1925) and also resembled the granulosa lutein cells described by Leeson and Leeson, (1970). They were termed LPC (large pale-staining cells, see following section 5.1.1.2). However, in 2 of 4 rabbits, a few small areas in the cortex of the ovaries had normal morphology and normal numbers of secondary and primary follicles.

Degenerate follicles were often seen in both the MV-ZPB and MV-HA infected groups. These were characterised by shrinkage and the presence of degenerate granulosa cells (clusters of cells with dark-stained nuclei) in the follicles (Figure 5-12).

More degenerate follicles were seen in the day 15 groups than the day 30 groups. Although there were no obvious differences between the MV-ZPB and MV-HA infected rabbits at day 15 (Figure 5-12) there were more degenerated follicles in the MV-ZPB infected rabbits at day 30. These results suggest that infection of the rabbits with recombinant myxoma virus caused secondary and tertiary follicle disruption, which peaked between day 5 and 15 post-infection. This was also the peak period of the IgG antibody response. The findings also suggest that the initial follicle loss is maintained for longer in the presence of antibodies to ZPB.

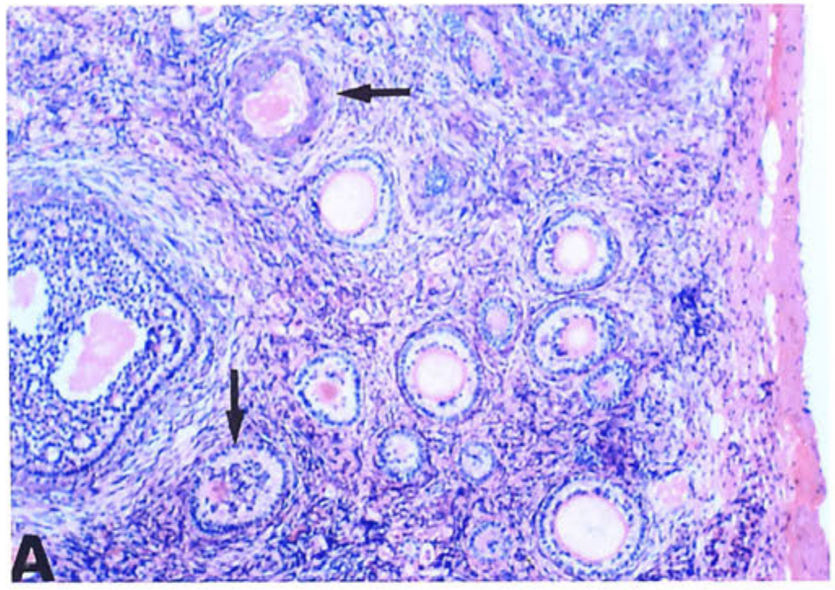
5.3.5.3 LPC accumulation in the infected ovaries

As mentioned above, an obvious change in MV-ZPB infected ovaries was the accumulation of LPC, notably in 3 of the 4 rabbits in the MV-ZPB D30 group. Most areas of the ovarian cortex and medulla were infiltrated with LPC (Figure 5-13). Vacuoles were present in the cytoplasm of most LPC (Figure 5-13) though in some areas these cells were more pink-stained and the vacuoles were hard to see. Accumulation of LPC appeared to be associated with follicle degeneration and loss with no follicles being found in the areas infiltrated with LPC (Figure 5-13).

LPC were not found in ovarian sections from uninfected controls. They were first seen in the medullas of 2 ovaries from the MV-ZPB D5 group and were found in greater numbers and more rabbits of the MV-ZPB D15 group. In the day 15 group, LPC were not only found in the medulla but were also seen in the cortex in some rabbits. Massive accumulation of LPC in both medulla and cortex were observed in most ovaries from rabbits in the MV-ZPB D30 group (Table 5-5). This suggests that accumulation of LPC may start at about 5 days post-infection and progressively increase until at least 30 days post-infection. Accumulation of LPC was also observed in the MV-HA infected rabbits at both time points but at a lesser intensity than in the corresponding MV-ZPB infected groups (Figure 5-11, Table 5-5). Since follicle loss also occurred in the MV-HA infected rabbits, this accumulation of LPC may be an expression of ovarian pathology following any major physiological insult which subsequently leads to follicle degeneration.

Figure notes are on the other side

Figure 5- 12. Follicle degeneration in ovaries from rabbits infected with recombinant MV (100x). Degenerate follicles (arrows) are present in ovaries from MV-ZPB infected rabbits at both 15 days (A) and 30 days (B) post-infection. Degenerate follicles are also present in ovaries of rabbits infected with MV-HA at both time points (15 day, C and 30 day, D).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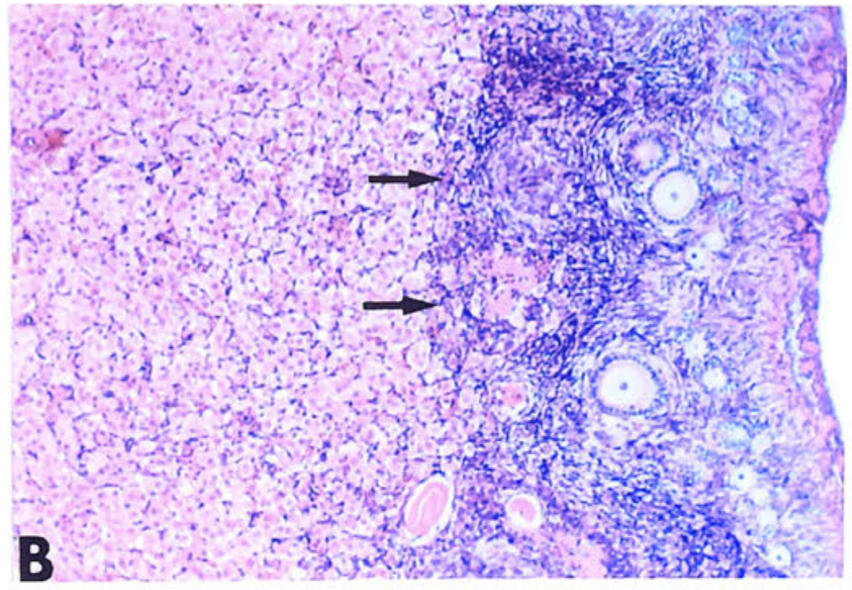
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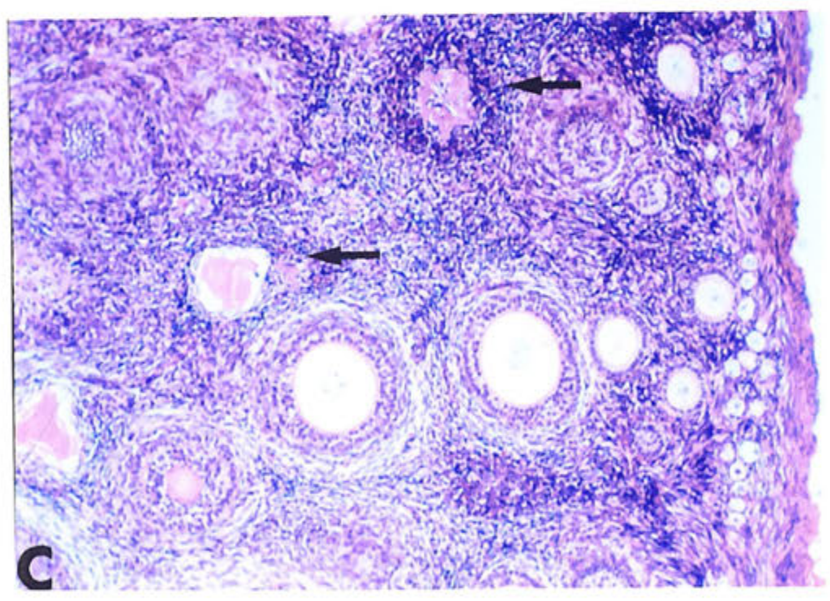
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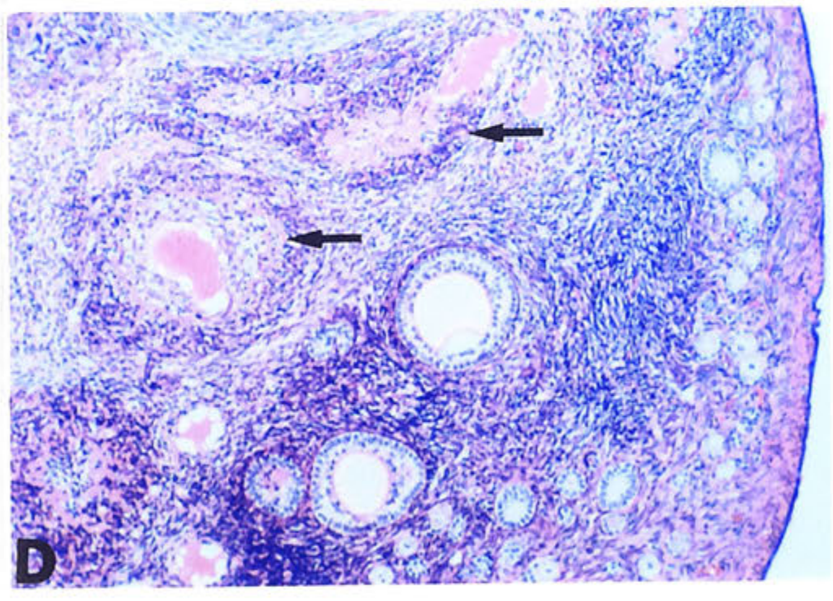
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Figure 5-13. The pattern of LPC accumulation in ovaries from rabbits infected with MV-ZPB. LPC first appeared as small cells close to degenerate follicles (A, arrow, 100x). LPC then increased in size and progressively occupied more areas in the cortex (B, C, Lc, 100x). As the area occupied by LPC expanded, the follicles degenerated at the edge (B, long arrow and C). Image D is a higher magnification (400x) of C showing the morphology of LPC which are large, pale-stained cells with vacuoles in the cytoplasm.

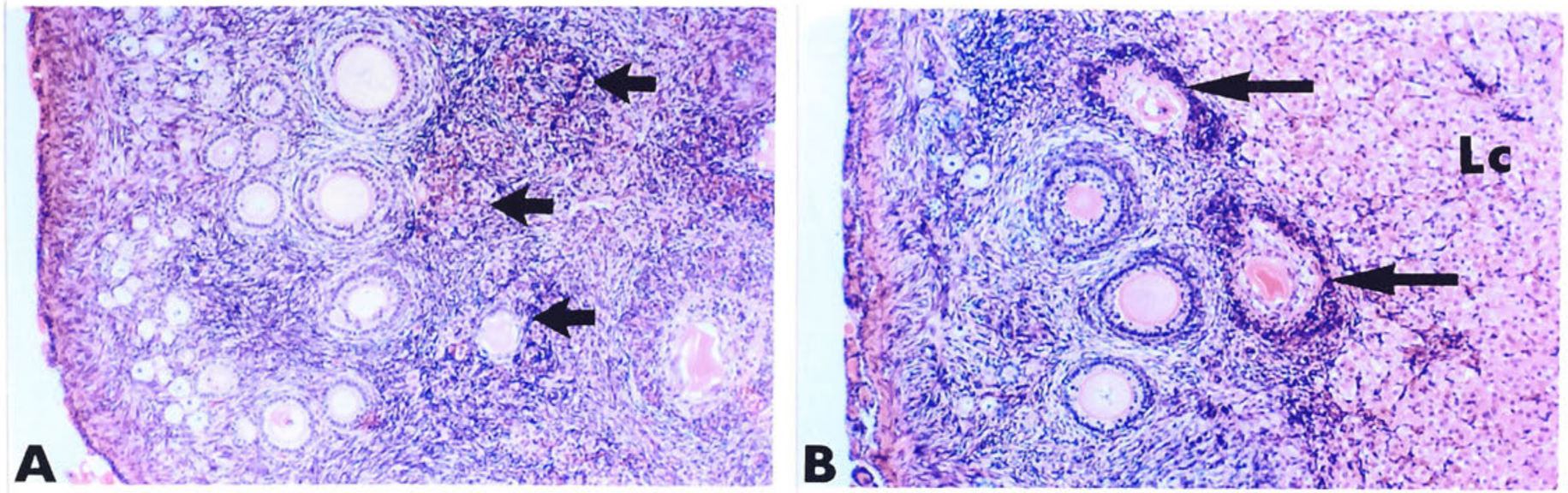
A
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Table 5- 5. The rate and intensity of LPC accumulation in the ovary

Groups	-	+	++	+++	++++
Control	4/4	0	0	0	0
MV-ZPB D5	2/4	2/4			0
MV-ZPB D15	0		3/4	1/4	0
MV-ZPB D30	0	0	0	1/4	3/4
MV-HA D15	0	2/4	0	1/4	1/4
MV-HA D30	0	2/4	1/4	1/4	0

Notes: The values are rabbit number. The accumulation of LPC was ranged as: -, no LPC in whole section. +: LPC were present in some area of the medulla. ++, LPC were present in most areas of the medulla but not in the cortex. +++, LPC were seen in most areas of the medulla and some areas of the cortex. ++++: LPC were present in the whole medulla and most areas of the cortex.

LPC could originate from two locations. Firstly, LPC could originate in the medulla, accumulate there and spread to the cortex because in the day 5 group, LPC were only seen in the medulla, whereas in the day 15 and 30 groups these cells were also present in the cortex. Secondly, LPC might form from degenerating follicles, because some isolated LPC-like cells were seen in degenerate follicles from both the day 15 and day 30 groups infected with either MV-ZPB or MV-HA (Figure 5-13).

5.3.5.4 Infiltration of lymphoid cells in the tertiary follicles

Leucocyte infiltration was examined earlier in the chapter by staining for B and T cells infiltrating into the ovary. To further examine this in H & E stained sections, the number of tertiary follicles that had lymphoid cells surrounding the oocyte was counted in one section from each rabbit (Table 5-6). No significant differences were found between MV-ZPB infected groups and their controls except MV-ZPB D15 against MV-HA D15 (Table 5-6). In the H & E stained sections, lymphocytes were identified by their morphology – dark stained nuclei, little cytoplasm and small, round, size and shape. While in some sections there was clearly a lymphocyte infiltration around some follicles, overall there was no difference between infected and control rabbits.

Table 5- 6. The number and ratio of tertiary follicles infiltrated with lymphoid cells in the ovarian sections

Groups	Infiltrated follicles	Infiltrated follicles / Total follicles
Controls	1.3±0.9	0.09±0.06
MV-ZPB D5	1.8±2.4	0.12±0.15
MV-ZPB D15	2.8±1.3*	0.19±0.08*
MV-ZPB D30	1.3±1.5	0.09±0.11
MV-HA D15	0.8±0.9	0.05±0.06
MV-HA D30	2.3±0.9	0.13±0.04

Notes: the values are mean ± S.D for four animals. *: $P<0.05$ against MV-HA D15 group.

5.3.6 The relationship between follicle number and infertility rate

A significant reduction in follicle numbers at pre-ovulatory or tertiary stages was observed in the rabbits immunised with MV-ZPB (section 5.3.5.2). However, a previous study showed that only 25% females were infertile after immunisation with the same recombinant MV-ZPB (Kerr *et al.* 1999). This suggests that the reduction in pre-ovulatory or tertiary follicle numbers obtained may not be sufficient to cause infertility in most rabbits. In the same study, it was shown that the infertility rate increased to 80% after the MV-ZPB infected females were boosted twice with recombinant ZPB protein (Kerr *et al.* 1999). To explore a possible relationship between follicle loss and infertility rate, four female rabbits were infected with MV-ZPB and boosted twice with recombinant rabbit ZPB protein in Freund's incomplete adjuvant (Chapter 2, section 2.2.2.1) at 30 and 44 days post-infection. Blood and ovaries were collected at 58 days post-infection. ZPB-IgG titers were measured in serum and the immunological and histological changes in the ovaries were examined.

5.3.6.1 IgG antibody in serum and in the ovary of boosted rabbits

The mean serum IgG antibody titer at 58 days in the boosted rabbits was 2.7 ± 0.51 (mean ± S.D), which was not different from that of the MV-ZPB D30 group and was

much lower than the antibody titer in the study of Kerr et al, (1999). A possible explanation for this is that the batch of recombinant rabbit ZPB used in the current study contained a lower ZPB protein concentration than was indicated. When the same protein solution was used for ELISA only a very low level of antibody binding was obtained and a similar result was obtained with immuno-blotting (data not shown). Taken together, these results suggest that this protein solution contained a lower ZPB protein concentration than the supplier indicated. This boost experiment could not be repeated because of time limitations.

The location of IgG antibody in the ovaries was examined in frozen sections using immunofluorescence as described above (section 5.3.2). Stronger IgG staining than that of the MV-ZPB D30 group (Figure 5-3) was seen on the ZP of oocytes in most secondary or tertiary follicles of the four rabbits (Figure 5-14). The ZP of oocytes in primary follicles were not IgG positive. As in rabbits infected with MV-ZPB alone (section 5.3.2.2 and 5.3.2.3), only weak binding of IgM or IgA to the ZP was seen in a few follicles. This was probably non-specific.

5.3.6.2 CD43 and KEN-5+ cells in the ovary of boosted rabbits

Ovarian sections from the boosted rabbits were stained for both CD43+ and KEN-5+ as described in section 5.3.4. There were increases in both CD43+ and KEN-5+ cell numbers in two of the four boosted rabbits compared to the uninfected controls. These cells were located closely around the follicles (Figure 5-15). The other two boosted rabbits were not different from the uninfected controls described in section 5.3.4.2.

5.3.6.3 Ovarian weight and pre-ovulatory follicles in boosted rabbits

The mean ovarian weight of the boosted group was 0.14 ± 0.01 for right ovaries and 0.14 ± 0.02 for left ovaries, significantly lower ($P<0.01$) than in uninfected controls (Table 5-2) but not different from the MV-ZPB D30 group or other infected groups. The mean pre-ovulatory follicle number in these ovaries was 2.3 ± 1.5, significantly lower ($P<0.01$) than that of the uninfected controls (6.5 ± 1.7) but not different from the MV-ZPB D30 group (2.3 ± 1.5, Table 5-3).

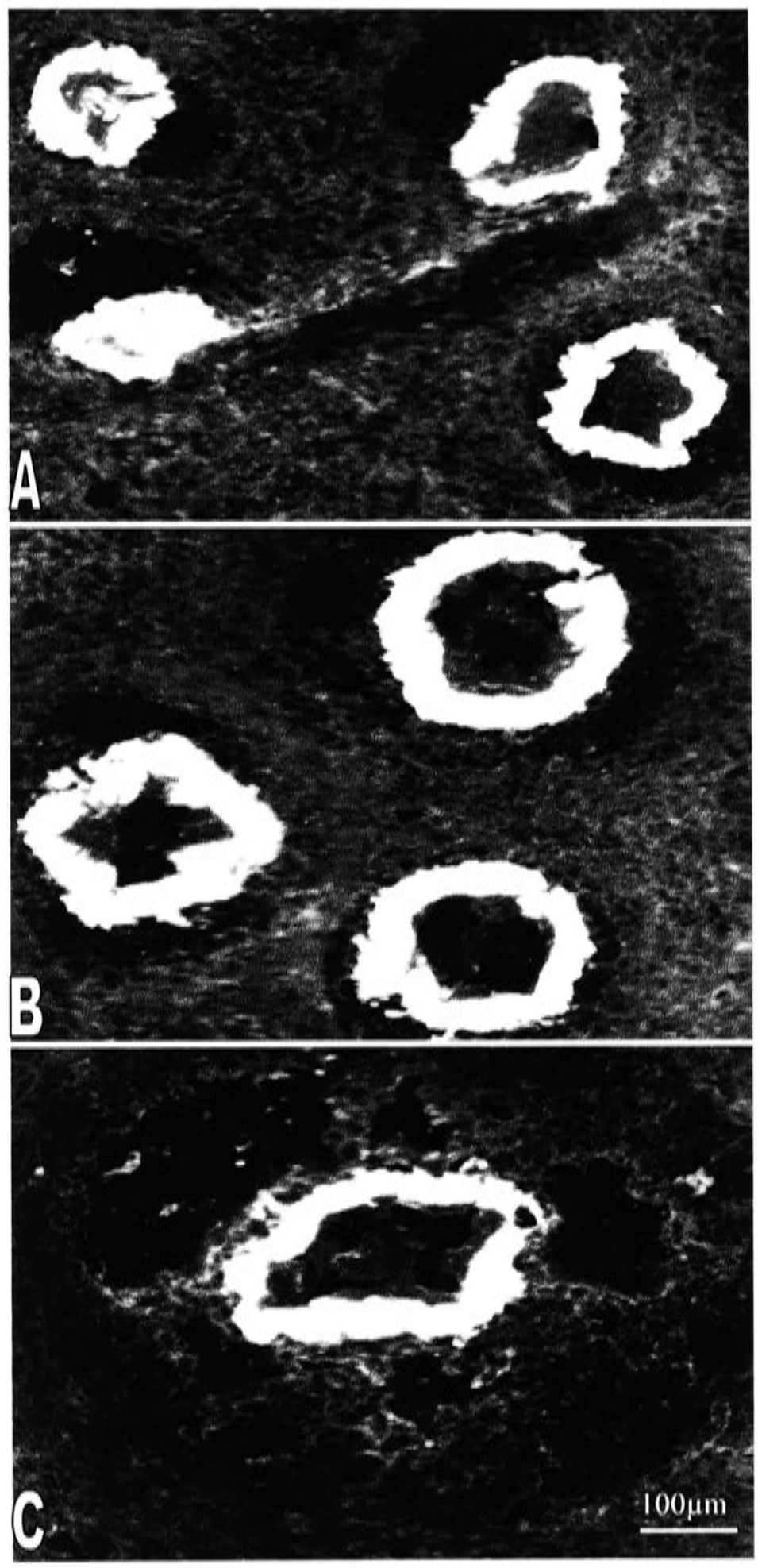


Figure 5- 14. IgG localisation in ovaries of rabbits infected with MV-ZPB and boosted with recombinant ZPB protein. Strong IgG staining was localised on ZP of oocytes in secondary (A and B) and tertiary (C) follicles from rabbits infected with MV-ZPB and boosted with recombinant ZPB protein. All images are at the same magnification.

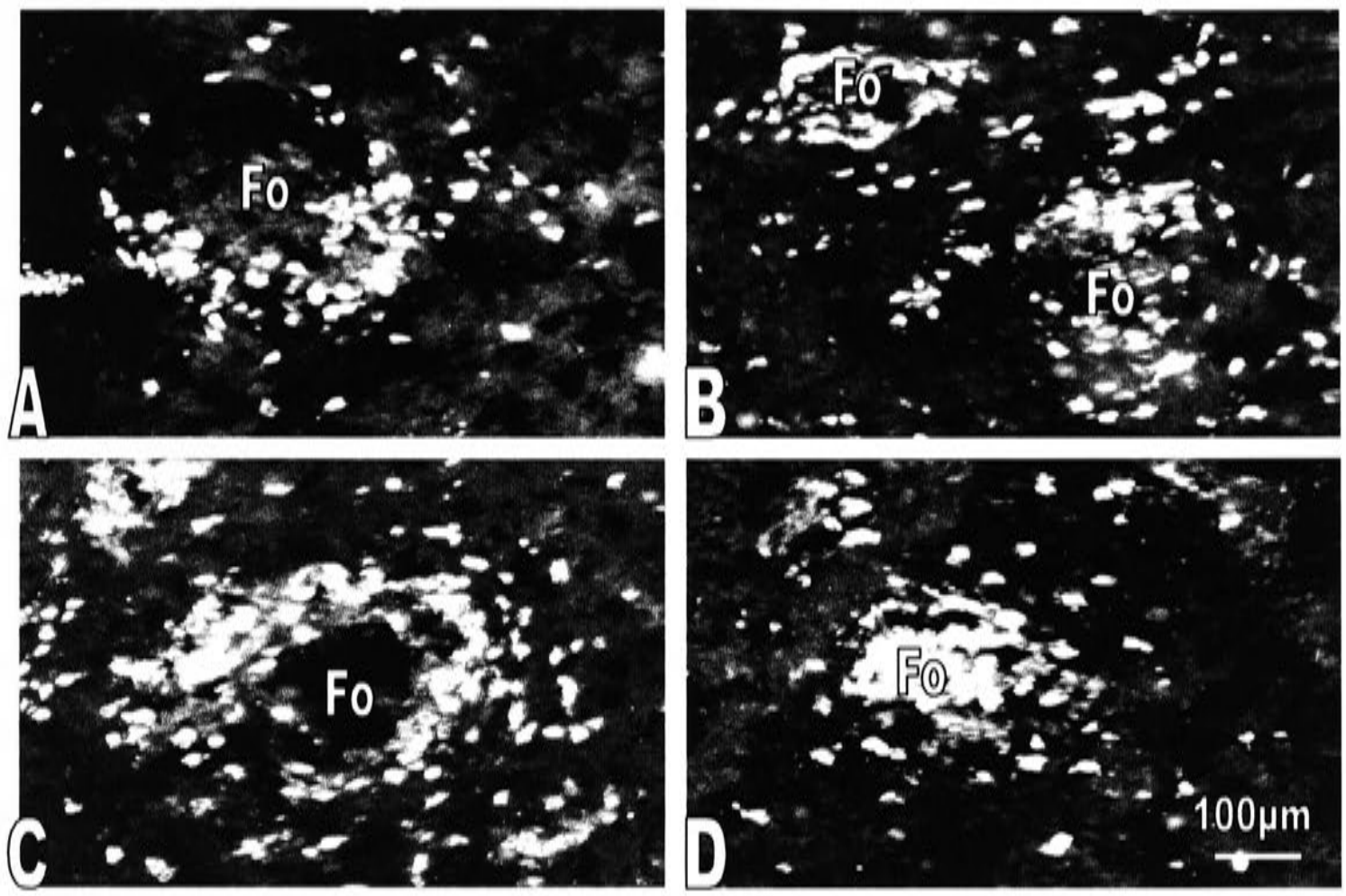


Figure 5- 15. KEN-5+ and CD43+ T cells in ovaries of rabbits infected with MV-ZPB and boosted with recombinant ZPB protein. The images A and B show that KEN-5+ cells are present in the areas close to ovarian follicles (Fo) in boosted rabbits. C and D show that CD43+ T cells are also present around the follicles (Fo). All images are at the same magnification.

5.3.6.4 Follicle loss and LPC accumulation in the ovary of boosted rabbits

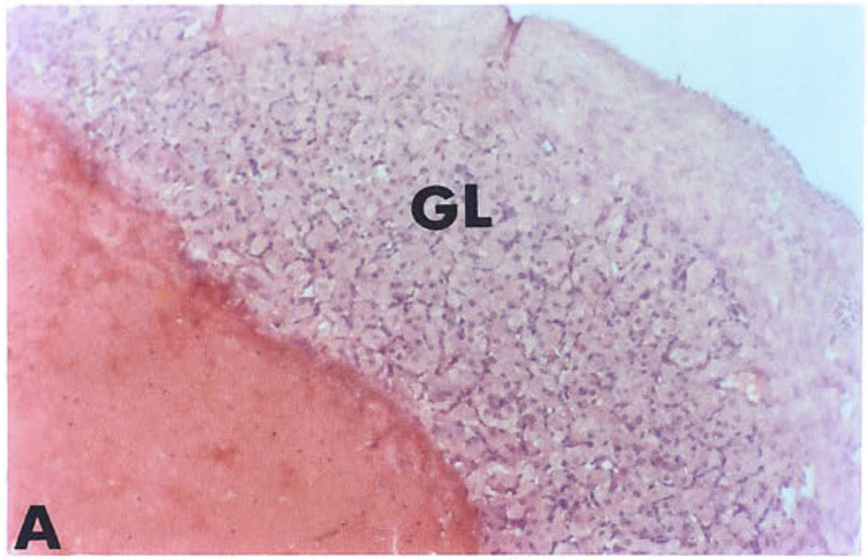
The number of tertiary follicles for the individual boosted rabbits was 6, 4, 10 and 27. The mean ± S.D was 12 ± 10.5. This number was not statistically different from the uninfected controls and the MV-HA D30 group. However, the reduction in follicle number in three of the four boosted rabbits is worth noting. The mean numbers of primary and secondary follicles in the four rabbits were 15.75 ± 11.1 and 16 ± 6.4, respectively, significantly lower ($P<0.05$ for both) than that of uninfected controls. Numbers of primordial follicles in the 3 rabbits with fewer tertiary follicles were also reduced compared with uninfected controls and severe LPC accumulation was also observed in these rabbits (Figure 5-16). However, less LPC accumulation was seen in the ovary with the high number of tertiary follicles.

5.3.7 Granulosa lutein cells

As mentioned above, LPC had some resemblance to granulosa lutein cells which are described as large, pale-staining cells with large vesicular nuclei. They are derived from granulosa cells and are found in ovulated follicles (Leeson and Leeson, 1970). Because ovulation in rabbits occurs only after mating, granulosa lutein cells are usually not observed in ovaries from unmated rabbits. To compare granulosa lutein cells in ruptured follicles with LPC found in ovaries from infected rabbits, ovulation was induced in two rabbits by hCG injection (Chapter 2 section 2.2.2.2) and another two rabbits were mated to induce ovulation. The ovaries were collected 5 days after ovulation for histological examination. Granulosa lutein cells at different developmental stages were observed in these ovarian sections (Figure 5-16). Although some granulosa lutein cells at later developmental stages looked similar to LPC, overall they were smaller in size and lacked lipid droplets in the cytoplasm (Figure 5-16). In addition, the granulosa lutein cells were restricted to the follicles and did not appear to be invading other tissues. Furthermore, LPC accumulation occurred in the absence of ovulation which is the normal trigger for their formation.

Figure notes are on the other side

Figure 5-16. Comparison of granulosa lutein cells in ovulated follicles and LPC (100x). A, B and C show granulosa lutein cells (GL) at different stages of follicle development. Image A shows an early development stage of GL where large amounts of blood were still present in the center of the follicles. Image B shows the next stage of GL where the blood has almost disappeared. Image C shows a later stage of GL where no blood can be seen in the follicle. Image D shows LPC (D, Lc) in ovaries from the boosted group.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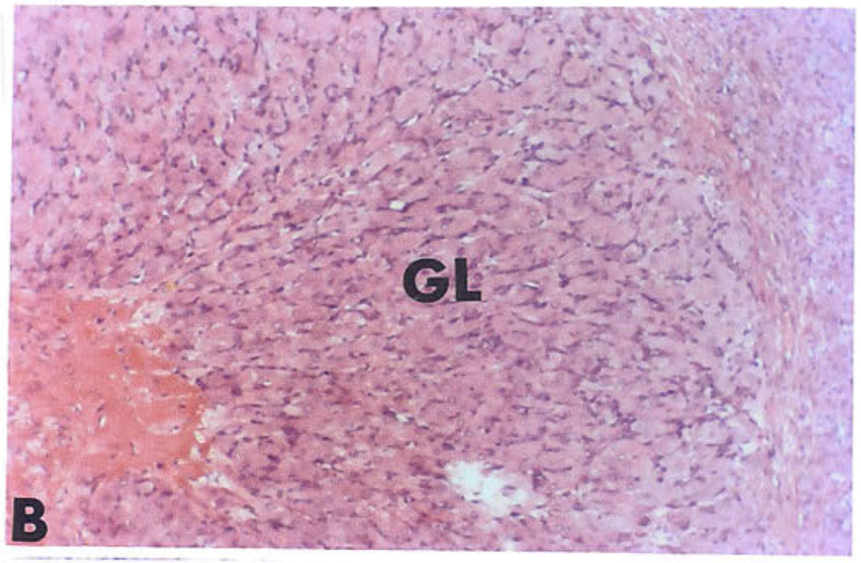
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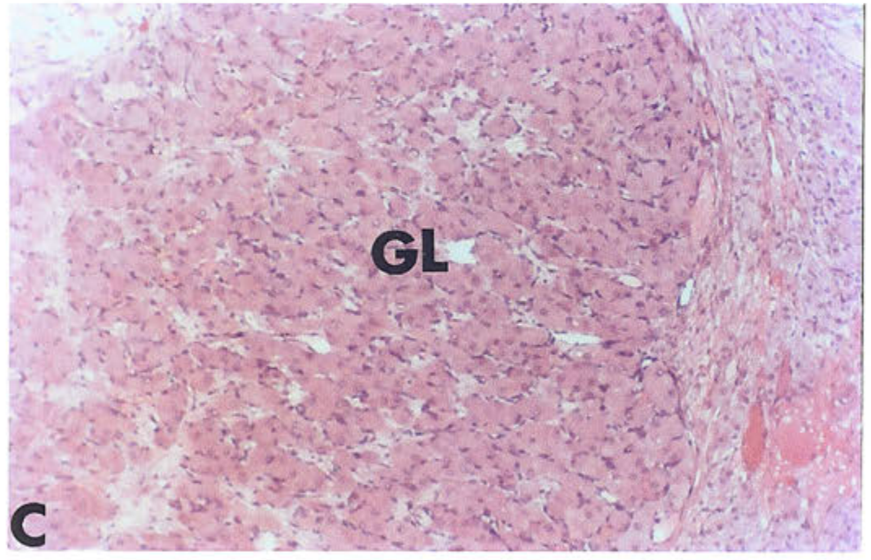
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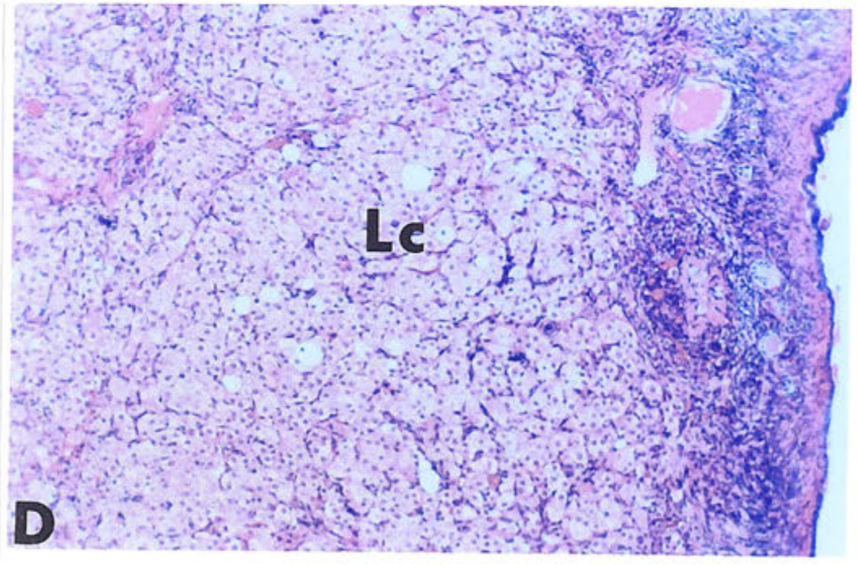
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5.4 Discussion

5.4.1 Immune response to ZPB in the ovary and immune tolerance to ZPB

It is normally difficult to induce an immune response to a self-antigen because of immune tolerance. The mechanism of controlling self-tolerance can be at a central level such as negative selection and clonal deletion of autoreactive T and B cells before or immediately after birth –or by peripheral suppression of autoreactive lymphocytes (Miller, 2001; Huang and MacPherson, 2001).

ZPB is a self-antigen, which is not produced in rabbits until about a month after birth (Kim Baker, PhD thesis, ANU, 1996). The tolerance of female rabbits to ZPB can be observed by immunising female and male rabbits with ZPB protein in Freund's complete adjuvant. Male rabbits mount a strong antibody response to the first (primary) immunisation whereas female rabbits generally require several boosts to generate high antibody titers. High antibody titres are also associated with a strong delayed type hypersensitivity response (Kerr *et al.*, 1999). Thus, tolerance can be overcome in female rabbits by repeated immunisation with ZPB protein indicating that autoreactive T and B cells are present but are presumably controlled at the peripheral level.

In contrast, female and male rabbits immunised with recombinant myxoma virus expressing ZPB (MV-ZPB) have an essentially identical antibody response to ZPB indicating that delivery of the antigen encoded in the virus overcomes the initial tolerance reactions in female rabbits. However, in both genders, the ZPB antibody response was not sustained following MV-ZPB immunisation (Kerr *et al.*, 1999).

In this chapter, antibody titers to ZPB peaked at 15 days in female rabbits immunised with MV-ZPB but then dropped away, a result essentially similar to that of Kerr et al. (1999). Kerr et al. (1999) found no pathological changes in the ovaries of rabbits immunised with MV-ZPB but these were only examined 90-100 days after immunisation. In the present study, ovaries were examined at 5, 15 and 30 days after immunisation with MV-ZPB and stained for antibody, B and T cell responses in order to understand the early effects of immunisation at the ovarian level.

In the current study, an IgG antibody response to ZPB was induced in serum of female rabbits infected with MV-ZPB at 15 and 30 days post-infection. This result confirms that an antibody response to the self-antigen ZPB can be elicited when ZPB is delivered by a recombinant virus (Kerr *et al.* 1999). Previous studies in rabbits immunised with either native rabbit ZP or *E. coli*-expressed ZPB were unable to demonstrate an antibody response to ZP proteins (Wood *et al.* 1981; Prasad *et al.* 1995). IgG antibody specifically bound to ZP of oocytes in ovaries both *in vivo* following immunisation with MV-ZPB (IgG localisation, Figure 5-2, 5-3) and *in vitro* (immuno-fluorescence assay, Figure 5-7). These results suggest that specific IgG to ZPB was induced in serum and that serum IgG had entered the ovarian follicle where it bound to ZP. Although IgM antibody could also freely transfer into the ovary from serum (Chapter 4 section 4.3.9), very little IgM was detected bound to the ZP. This may have been due to a lower titer of ZPB-IgM compared to ZPB-IgG in serum or to lower antibody affinity for ZPB. However, IgM serum titers were not measured here. As expected from the lack of a serum IgA response to MV-HA infection, no IgA binding to ZP of oocytes in the ovary was found. These results confirmed that IgG is the dominant antibody class in both the serum and the ovarian follicle following MV-ZPB infection. The binding of IgG to ZP, particularly to the ZP of oocytes in tertiary follicles, may prevent sperm from attaching to the oocytes and so block fertilisation after ovulation (Chapter 1 section 1.6.2.1). This ability of MV-ZPB to induce antibody that binds to ZPB indicates that MV-ZPB has the potential to be an immunocontraceptive vaccine. However, results published during the course of this study indicated that immunisation with MV-ZPB induced only transient infertility in 3 of 12 female rabbits. This contrasts with the effect of repeated immunisation with ZPB protein in adjuvant which induced higher antibody titers and long-term infertility in 75% of immunised rabbits (Kerr et al., 1999). In addition, compared with the serum antibody response to HA, a model strong antigen, delivered by the same recombinant virus (Chapter 4), the serum antibody response to ZPB was much lower and shorter-lived.

The mechanism of overcoming immune tolerance to self-antigens delivered by a recombinant virus is not fully understood. One possible explanation for the overcoming of self-tolerance to ZPB in the female rabbit is that the tolerance is due to peripheral suppression of ZPB reactive T cells. However it is possible that the viral protein-ZPB

complex could bind to B cells through their surface Ig specific for MV protein or ZPB or be taken up by APC. These cells could then present both ZPB and MV peptides on MHC-II molecules to T cells. MV specific T cells could then activate those B cells exhibiting both ZPB and viral peptides (Jackson *et al.* 1998). Alternatively, peripheral tolerance to ZPB might have been induced and maintained by immature dendritic cells that lack MHC II molecules and co-stimulation factors (Jonuleit et al. 2001; Mahnke et al. 2002). When ZPB carried by the recombinant virus infects these cells (Best and Kerr, 2000), the viral antigenic peptides could help in their activation and maturation including the promotion of expression of MHC II and co-stimulation factors (Mahnke et al., 2002). These matured dendritic cells may then develop the capacity to present ZPB peptides to T or B cells and induce an immune response to ZPB. A more general explanation is that the viral infection created an inflammatory micro-environment (enriched with proinflammatory molecules such as cytokines) which non-specifically increased immune responsiveness, antigen presentation by APC and the responsiveness of auto-reactive T and B cells to ZPB.

A previous study showed that mouse ZPC delivered by a recombinant mouse poxvirus induced both an antibody response to ZPC and infertility in female mice (Jackson *et al.* 1998). Similar results were obtained with murine cytomegalovirus expressing mouse ZPC (Chambers et al., 1999). Recombinant vaccinia virus, which delivered a mouse tumour antigen, was also shown to be able to overcome self-tolerance and induce an autoimmune response that caused the destruction of tumour cells (Overwijk *et al.* 1999). These data collectively confirm the conclusion that the delivery of self-antigen by a recombinant virus has the ability to overcome immune tolerance and induce an immune response.

A T cell response in the ovary was detected in only one of four rabbits in both the day 5 and the day 15 groups. At this stage, it is not possible to prove that these T cells actually reacted with ZPB. Since a B cell response, as measured by antibody production, was mounted to ZPB but then quickly waned, it seems that a good B memory cell response was not obtained. This lack of a good memory response could be explained by tolerance at the level of T cell help.

This raises the question of how a B cell response to ZPB was initially generated. One hypothesis is that B cells bind to viral proteins complexed with ZPB via a ZPB antibody on the B cell surface. The antigen complex is internalised and processed. This can provide two sets of peptides, one derived from viral proteins, the other from ZPB, to interact with T cells. Viral proteins can activate a T cell response and stimulate B cell responses to both the viral antigen and ZPB, probably by cell-cell interaction and producing T cell cytokines in the micro-environment. However, ZPB peptides did not activate T cell responses including T memory cells to ZPB. In the absence of T cell help, B cell response to ZPB was short-lived. APC could also fail to activate T cell responses to self-antigen (Roitt, 1997) or only activate a weak T cell response to ZPB but could stimulate a strong T cell response to the viral antigen. In the absence of T helper cells, this would result in lower and shorter-lived IgG responses to ZPB. Another possibility is that B cell tolerance to ZPB is maintained by self-antigen eliminating or inactivating some self-reactive B cells (Goodnow, 1992). The fact that the overall antibody response to ZPB was lower than that to HA might indicate that fewer B cells were involved in the response.

5.4.2 Follicle loss and infertility

5.4.2.1 A potential mechanism for IgG-mediated tertiary or secondary follicle disruption

Reduction in follicle numbers following ZP immunisation has frequently been reported (Sacco, 1987; Henderson *et al.* 1988; Skinner *et al.* 1996), possibly reducing the ability of the ovary to produce both oocytes and ovarian hormones (Skinner *et al.* 1984). Significant loss of follicles at the pre-ovulatory or tertiary stage was also observed in the current study after MV-ZPB infection. This follicle loss could be attributable to a combination of specific factors such as antibody or specific T cell responses and non-specific factors such as fever resulting from the virus infection. Infection with MV-ZPB caused mild myxomatosis and fever (Kerr *et al*, 1999). Mild myxomatosis was also seen in the current study. This clinical disease might have physically affected the development or maintenance of ovarian follicles. This was suggested by the significant ovarian weight loss in infected rabbits at both 15 and 30

days post-infection and the presence of more degenerate follicles in ovaries of infected rabbits compared to uninfected controls.

However, viral infection with MV-HA did not cause significant follicle reduction at either 15 or 30 days post-infection. Histological examination also indicated that ovarian pathology in MV-HA infected rabbits was not as severe as that in MV-ZPB infected rabbits especially in the day 30 group with less accumulation of LPC in the cortex area. Furthermore, a previous study showed that infection with the parent strain of MV-ZPB (strain Uriarra) showed no effect on fertility (Kerr *et al.* 1999). This suggests that the viral infection has only a limited effect on ovarian function and fertility.

Because rabbit IgG cannot fix complement (Mage, 1998) and IgG was the dominant antibody class bound to the ZP in the ovary, it does not appear likely that IgG induced complement damage in the ovary. As mentioned above, only one of four infected rabbits showed a T cell response in the ovary whereas reduced numbers of both pre-ovulatory and tertiary follicles were observed in all four rabbits in the MV-ZPB D30 group. This result suggests that T cell responses may not play a major role in follicle loss. However, T cells were present in one of the four infected rabbits where they may damage follicles by producing cytokines or other factors.

After excluding viral infection and T cell responses, IgG to ZPB remains as the main factor responsible for follicle loss (Skinner *et al.* 1984; Kerr *et al*, 1999). This was supported by the fact that IgG binding was to secondary and tertiary follicles which were also the follicles that were undergoing degeneration or reduction in number compared with control ovaries. This is also consistent with the development of rabbit ZP, in which ZP proteins first appear at the primary follicle stage (but may not be detectable by immunofluorescent staining) and are much more developed in secondary and tertiary follicle stages (Skinner, 1996). IgG was also suggested to play a major role in follicle loss and ovarian dysfunction in rabbits immunised with porcine ZP (Skinner *et al.* 1984) and antibody titer to ZPB was correlated to infertility in rabbits immunised with ZPB protein (Kerr *et al*, 1999).

How could IgG antibody cause follicle disruption? One explanation suggested by Dunbar (1989) is that antibody binding to ZP might block communication between

oocytes and granulosa cells. Consequently, the oocytes would not exchange essential signals with the microenvironment, which might result in oocyte degeneration. Equally, granulosa cells would also lack signals from oocytes and therefore undergo degeneration or luteinization. This combined failure of signalling between follicle and oocyte would lead to follicle disruption. Eppig *et al.* (1997) have shown that oocytes can control the differentiation of granulosa cells by secreting paracrine-signalling factors which confirms communication between oocyte and granulosa cells and supports the signal disruption hypothesis. Nevertheless, this hypothesis remains to be tested. Another possible explanation is that antibody could combine with newly formed ZP and change ZP structure thus affecting oocyte development that would result in granulosa cell and follicle disruption.

5.4.2.2 Is there a relationship between follicle loss and infertility

In the current study, the number of pre-ovulatory and tertiary follicles for the four rabbits in the MV-ZPB D30 group was significantly reduced. However, in the previous study, only 3 out of 12 (25%) MV-ZPB infected rabbits were infertile when they were mated at 34 days post-infection (Kerr *et al.* 1999). This suggests that the significant loss of pre-ovulatory and tertiary follicles may not necessarily correlate to infertility. Kerr et al. (1999) showed previously that if the female rabbits infected with MV-ZPB were boosted twice with recombinant ZPB protein, the infertility rate increased to 80% (Kerr *et al.* 1999). Similar immunological and histological changes to those in the MV-ZPB D30 group were seen in ovaries from a group of rabbits boosted in the same way as Kerr et al. (1999). However, compared with the MV-ZPB D30 group the boosted group had significantly fewer primary and primordial follicles, consistent with the lower fertility reported by Kerr *et al.* (1999). These studies suggest that a high infertility rate may require loss of primordial or primary follicles as well as pre-ovulatory or tertiary follicles. Significant primary and primordial follicle reductions and high infertility rates were reported by previous studies in rabbits immunised with porcine ZPC (Skinner *et al.* 1984; Jones *et al.* 1992). These data support this conclusion.

How is the reduction of primary and primordial follicle reduction linked to a higher infertility rate? One obvious possibility is that once the follicles at early developmental stages are disrupted in the ovary, the entire follicular pool is depleted and the ovary

therefore cannot produce oocytes or normal secretion of hormones and this leads to infertility. In contrast, in the case of the MV-ZPB D30 group, primary and primordial follicles remained basically normal so although there would be an initial level of infertility following the loss of secondary and tertiary follicles, subsequent development of primary follicles would result in the infertility being transient. However, in the current study IgG staining was not detected in primary and primordial follicles in the ovarian sections from either MV-ZPB infected or boosted groups and primary or primordial follicle reduction did not correlate with tertiary follicle reduction. This result supports the idea that primary and primordial follicle reduction is more likely to be a result of longer-term ovarian pathology or dysfunction including LPC accumulation and possible endocrine changes (see also following section 5.4.3) rather than a direct antibody reaction. From this point of view, the reduction of early stage follicles should occur after tertiary or secondary follicle reduction at later time points after infection.

5.4.3 Accumulation of LPC in infected ovaries

As well as a reduction in secondary and tertiary follicles, another obvious histological change in ovaries from MV-ZPB infected rabbits was the accumulation of LPC in both cortex and medulla. This was more obvious at later stages of MV-ZPB infection (the day 30 group) and in the boosted rabbits. Ovaries showing substantial LPC accumulation also showed extensive follicle loss. However, the reasons for LPC accumulation, their effects and the relation between LPC accumulation and follicle reduction or infertility are unknown.

Two cell-types, granulosa lutein cells (Leeson and Leeson, 1970) and expanded interstitial cells (Hammond and Marshall, 1925; Leeson and Leeson, 1970) are similar in description to LPC. The formation of LPC from granulosa cells is consistent with their origin close to degenerating follicles and with the possibility that antibody binding to the ZP results in an interruption of signalling between oocyte and granulosa cells. This latter might well result in atresia of the oocyte and aberrant development of the granulosa cells. However, careful examination of granulosa lutein cells (Figure 5-16) suggested that they had different cytological characteristics from LPC (Figure 5-13 and 5-16). The possibility that LPC could be expanded interstitial cells is supported by the

observation that LPC are first seen at sites where small follicles have degenerated. In humans, expanded interstitial cells can be seen in normal ovaries as a result of follicle degeneration but massive accumulation of interstitial cells is thought to result only from extensive follicle disruption (Leeson and Leeson, 1970). In rabbits, massive accumulation of LPC could also follow extensive follicle disruption by antibody. Further investigation will be necessary before any decision between these two alternatives, or indeed whether either is correct, can be made.

LPC have also reported in other studies in rabbits (Kerr *et al.* 1999) and mice (Jackson *et al.* 1998) where they have been described as luteinised cells. Further study is needed to understand LPC, especially in the endocrinological aspects.

5.4.4 Summary

Although recombinant myxoma virus has the ability to overcome self-tolerance to delivered antigens immune tolerance still existed to some extent compared to foreign antigens delivered by the same virus. The follicle disruption and ovarian pathology in the ovary following MV-ZPB infection is more likely to relate to IgG-mediated follicle degeneration than to a T cell response. High and long lasting levels of serum antibody (IgG) are needed to disrupt the primary and primordial follicles, which seem more important to higher infertility rate than disruption of secondary and tertiary follicles. From this point of view, the antigenicity of ZPB needs to be enhanced to achieve a higher infertility rate if ZPB is to be delivered by a recombinant myxoma virus.

CHAPTER 6: FINAL DISCUSSION

6.1 Introduction

As a critical step in developing a viral vectored immunocontraceptive vaccine for rabbit fertility control, this program focused on understanding the immune response in the female rabbit reproductive tract to antigens delivered by recombinant myxoma viruses. Recombinant myxoma virus did not induce high IgG and IgA responses to a model antigen (HA) in the mucosal compartments of the reproductive tract. However, strong IgG responses to HA were always induced in serum and the antibody could transfer freely from serum into the ovarian follicular fluids. If antibody is critical for immunocontraception, the fact that only low levels of antibodies were induced in the mucosal part of the reproductive tract whereas high levels of antibodies were induced in the ovarian follicles indicated that the ovary was a more appropriate target for immunocontraception if the antigen was to be delivered by recombinant MV.

Infection of female rabbits with MV-ZPB overcame self-tolerance to ZPB and induced an IgG response to ZPB. The antibody bound to the zona pellucida of oocytes in the ovary and this was associated with a significant reduction in the number of pre-ovulatory or tertiary follicles and other ovarian pathology including extensive accumulation of large pale-staining cells (LPC). This result, together with the low antibody response to MV-HA infection in the reproductive tract confirms that the ovary is a better target for immunocontraception delivered by a recombinant myxoma virus than is the mucosal compartment of the reproductive tract. However, although here all rabbits showed a similar loss of pre-ovulatory or tertiary follicles (Chapter 5), in a previous study immunisation with MV-ZPB led to only 25% infertility (Kerr *et al*, 1999). But boosting with ZPB protein was followed by a loss of primary or primordial

follicles that was correlated with high infertility (Kerr *et al*, 1999). Following boosting in the current study, there was also a loss of primary and primordial follicles. In this chapter, the implication of these results and the possible mechanisms behind them will be discussed. Some possible future experiments will also be outlined.

6.2 The Immune response to antigens delivered by a recombinant MV and the selection of immuno-contraceptive target antigens

6.2.1 IgA antibody response

It has been demonstrated in rabbits that, following immunisation with semen or sperm proteins, IgG and IgA to sperm antigens in the reproductive tract fluids were the main contributors to blocking fertilisation (Menge and Lieberman, 1974). Consequently, it has been the prevailing view that induction of effective IgG and IgA responses in the reproductive tract by the recombinant MV would be essential for developing viral vectored immunocontraception targeted at sperm antigens. However, studies with the model antigen delivery system MV-HA, or with the self-antigen expressing MV-ZPB have proved that MV is not an effective vector for inducing IgA antibody responses in rabbits.

Infection of female rabbits with MV-HA by intradermal inoculation (the natural infection route of the virus) was inefficient at inducing specific IgA responses in serum or reproductive tract fluids (Kerr and Jackson, 1995; Chapter 4). Infection of female rabbits with MV-ZPB confirmed this (Chapter 5). These results suggest that by its natural route of infection the virus is not effective at inducing an IgA response. Infection of female rabbits with MV-HA through a common mucosal inductive site (intranasal) also failed to induce an IgA response in serum or a high IgA response in the reproductive tract fluids and nasal washings (Chapter 4). An earlier study in rabbits showed that intra-tracheal immunisation with either live or inactivated influenza virus induced an IgA antibody response to HA in both serum and respiratory secretions (Kasturi and Hannoun, 1977). However, HA encoded by the recombinant MV did not

induce IgA suggesting that delivery by recombinant myxoma virus may interfere with generation of IgA responses. In the mouse, however, intranasal infection with a recombinant adenovirus expressing HSV-1 was effective at inducing IgA responses to the antigen in serum and vaginal secretions and B cell homing to the reproductive tract was also induced (Gallichan *et al.* 1993; Gallichan and Rosenthal, 1995; Gallichan and Rosenthal, 1998). Clearly, recombinant myxoma virus is less effective at inducing an IgA response in the rabbit than the recombinant adenovirus is in the mouse.

Based on the numbers of potential antigen-presenting MHC-II positive cells present, the rabbit vagina has more potential than other regions of the reproductive tract to present antigens and induce a local immune response (Chapter 3), probably through antigen presenting cells migrating to local draining lymph nodes (McGhee et al. 1994; 1999). Intra-vaginal immunisation of rabbits with DNA vaccines induced IgA antibody responses in vaginal washings (Schreckenberger *et al.* 2000). However, intra-vaginal infection with MV-HA did not induce an IgA response to either MV or HA in either the circulation or local mucosa even though this infection led to the replication of the virus in vaginal MHC-II+ cells and a strong IgG antibody response in the circulation (Chapter 4). Collectively, these data suggest that MV is not an effective vector for inducing an IgA antibody response in rabbits. This effect was not limited only to the vectored antigen; IgA antibody to MV was also not detected in either the circulation or reproductive tract fluids following mucosal infections with MV-HA (Chapter 4). However, rabbits are capable of mounting an IgA response to various antigens (Menge and Lieberman, 1974; Winchell *et al.* 1997; 1998; Schreckenberger *et al.* 2000) including HA (Kasturi and Hannoun, 1977), confirming that the absence of an IgA response in the present study is probably attributable to the mode of delivery as a recombinant MV and not any intrinsic lack of ability to mount an IgA response in the rabbit.

Compared with other species like mice and human, that have only one or two IgA isotypes, rabbits have 13 IgA heavy constant α-chain genes and 11 of which are expressed in rabbit tissues (Spieker-Polet *et al.* 1993; Spieker-Polet *et al.* 2002). Furthermore, in rabbits, different tissues express different isotypes with variable expression levels (Spieker-Polet *et al.* 1993; Spieker-Polet *et al.* 2002). It is not known

which isotypes are expressed in the reproductive tract fluids or at what levels they are expressed. It is also not known which of the 11 expressed rabbit isotypes of IgA are recognised by the anti-rabbit IgA polyclonal antibody used in the present study However, this antibody detected IgA in the reproductive tract fluids by ELISA and in the tissues by immunofluorescence. Furthermore, high titers of IgA were detected in the serum by immunodiffusion assay. It is possible that some IgA isotypes in the reproductive tract were not detected by this antibody. This would require further investigation.

6.2.2 IgG antibody response and transfer of IgG into the lumen of the reproductive tract

Recombinant MV is also not an effective delivery system for inducing a high IgG antibody titer in the reproductive tract fluids of rabbits. This was shown by the finding that only low levels of HA-IgG antibody were induced in the tract fluids and by comparison of the titers of IgG to HA in the serum and in the reproductive tract fluids following infections with MV-HA (Chapter 4). However, the recombinant MV consistently induced a high and lasting serum IgG antibody titer to HA (Chapter 4, Kerr and Jackson, 1995). In contrast to immunisation with recombinant adenovirus in the mouse, where intra-vaginal immunisation induced only low levels of serum IgG to HSV-I (Gallichan and Rosenthal, 1995), the recombinant MV was also effective at inducing a strong IgG response to HA in serum following intra-vaginal infection (Chapter 4).

The IgG present in female reproductive tract fluids is considered to be mainly derived from serum (Parr and Parr, 1996). For example, in mice the IgG titer in reproductive tract fluids was more than 10% of the IgG titer in serum (Gallichan and Rosenthal, 1995; Johansson *et al.* 1998). However, in the present study only about 1% of the serum IgG level was present in the luminal fluids after MV-HA infection (Chapter 4). In previous studies in rabbits IgG antibody was either not detected in the reproductive tract fluids (Edwards, 1960; Shapiro, 1971) or was detected at only 4 % of the levels in serum (Oliphant *et al.* 1977). Thus compared with the mouse, the rabbit is less efficient at transferring IgG into the reproductive tract. The oestrogen dominance in

the female rabbit reproductive tract before mating could account for the low IgG transfer efficiency; in rats, IgG levels in reproductive tract fluids were low at stages of the oestrous cycle when oestrogen concentration are high (Wira and Sandoe, 1980, Chapter 1 section 1.5.3). However, induction of ovulation in rabbits did not significantly alter IgG levels in the luminal fluids (Chapter 4). These data indicate that IgG transfer into the female rabbit reproductive tract is not influenced by oestrogen and progesterone in the same way as in rodents, in which the fall in oestrogen concentration and rise in progesterone concentration following ovulation is accompanied by an increase in antibody levels (Chapter 1 section 1.5.3). The finding that recombinant MV did not induce a high level of IgA and IgG in the female rabbit reproductive tract fluids and that serum IgG did not effectively transfer into the luminal fluids indicate that the virus is unlikely to be a suitable vector to deliver antigens such as sperm proteins for immunocontraception.

In contrast to the mucosal part of the reproductive tract, IgG and IgM in serum can freely transfer into the ovarian follicular fluids of rabbits (Chapter 4; Symons and Herbert, 1971; Kille and Goldberg, 1979). In addition, IgG antibody to ZPB induced by MV-ZPB infection was shown to enter ovarian follicular fluids and bind to oocytes (Chapter 5). These results suggest that, unlike the epithelial cells in the tract, there is no barrier between ovarian follicular fluids and serum, at least for IgG transfer. The fact that recombinant MV is effective at inducing a strong systemic IgG antibody response, and that this antibody could freely enter the ovarian follicles and react to oocytes, indicates that the ovary may be a more appropriate target for immunocontraception.

6.2.3 A model of the immune response to recombinant MV

The antibody response to myxoma virus infection is generally considered to be T cell-dependent. Both IgM and IgG antibodies to HA and MV were produced following infections with MV-HA (Chapter 4). This indicates that both HA and MV protein peptides have been presented by professional APC to CD4+ T cells and that intact antigens have been recognised by B cells. The role of T cell help is indicated by the switching from IgM to IgG production by B cells that has occurred (Figure 6-1). MV-HA is highly attenuated allowing the infection to be rapidly cleared, which in other

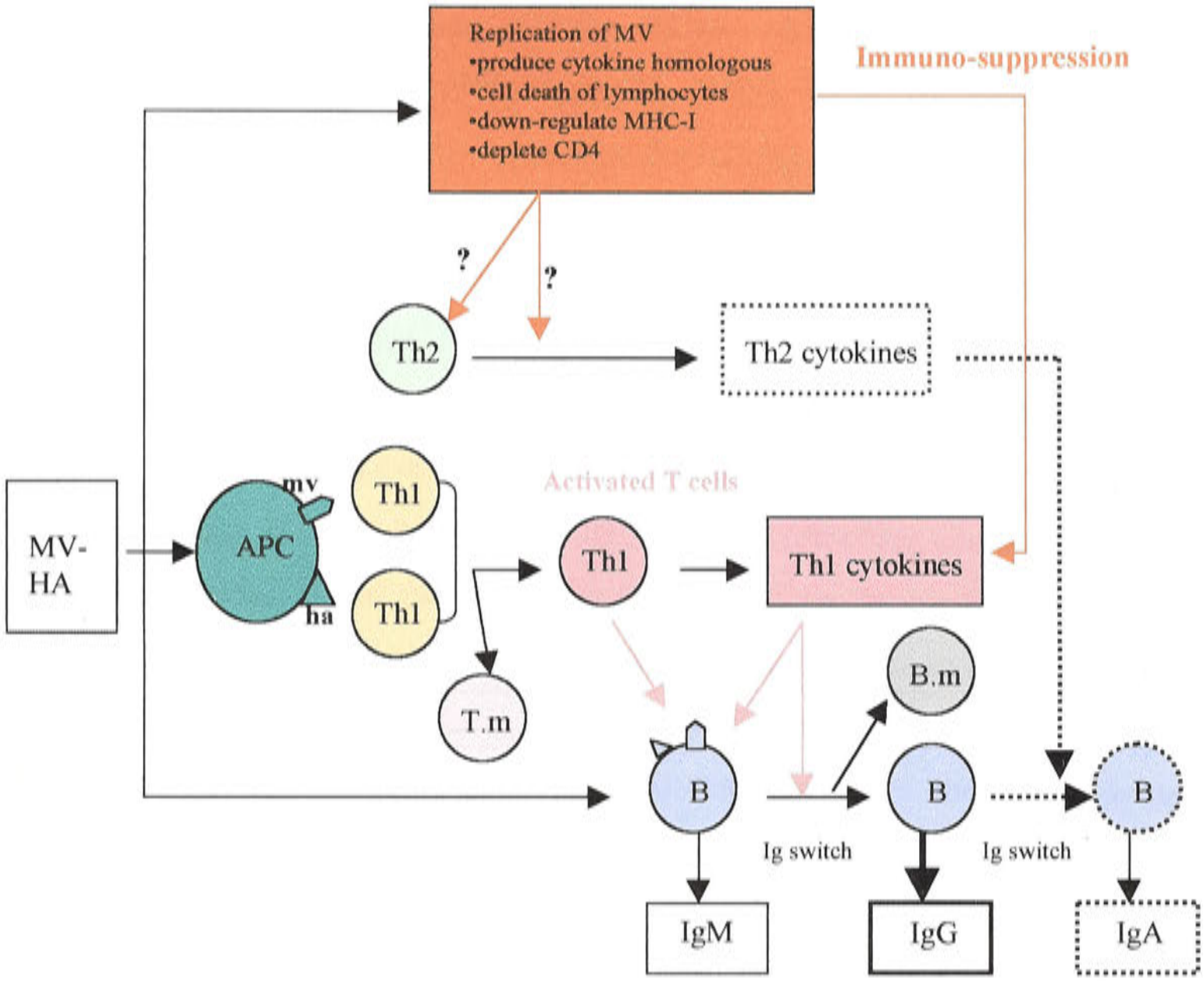

Figure 6-1. A model of antigen presentation and immune response to a strong antigen HA delivered by a recombinant myxoma virus (MV-HA) in the female rabbit. After infection of female rabbits with MV-HA, the APC at the inoculation sites (DC or LC in the skin; M cells in NALT and perhaps MHC-II + cells in the reproductive tract) take in and process the recombinant virus. Antigen peptides of both MV and HA are then presented complexed with MHC class II molecules at the APC cell surface (mv and ha). CD4+ T cells (possibly mainly Th1) recognise the peptide-MHC-II complex by T cell receptor (TCR) and are activated. Th1 cytokines (such as INF-γ, IL-2) are produced. In contrast, Th2 cell activation and Th2 cytokine production may be disturbed probably by an unclear mechanism. B cells can also bind the recombinant viral or HA antigen through cell surface antibody. The activated T cells (both MV and HA specific) and Th1 cytokines could help in stimulating strong HA and MV B cell responses including Ig isotype switch from IgM producing cells to IgG producing cells. The T and B memory (T.m and B.m) cells are also produced which ensure lasting antibody responses to MV and HA. The lack or absence of Th2 cytokines (such as IL-4, IL-5 and IL-6) would affect the Ig isotype switch from IgG producing cells to IgA producing cells and B cell homing to mucosal sites.

poxvirus infections requires an effective CD4+ and CD8+ T cell response as well as antibodies (Karupiah et al.,1996; Karupiah, 1998). Viral clearance implies that there was an effective T and B cell response to the infection. Based on the low levels of IgA to HA or MV, Ig isotype switching from IgG to IgA did not occur. It is known that Th2 cytokines such as IL-4, IL-5 and IL-6 are crucial in IgA isotype-switching and IgA production (Husband *et al.* 1994; Strober and Ehrhardt, 1994; Husband, *et al.* 1999). The weak IgA response could be due to a dominant Th1 response producing INF-γ and IL-2 rather than the Th2 cytokines, IL-4, IL-5 and IL-6. A strong Th1 response, together with production of IgG driven by a Th1 immune response, could explain the highly attenuated phenotypes of the recombinant virus and the low IgA to HA. Rabbits only produce one subtype of IgG (Knight, 1992) so further definition of the IgG subclass, as can be done for mice and humans, is not possible. Myxoma virus is postulated to inhibit Th1 responses (Best and Kerr, 2000) and down-regulates the rabbit inflammatory response (Nash, 1999) but this is probably not occurring efficiently with the highly attenuated MV-HA.

According to this model, recombinant myxoma virus is an effective vector to deliver an antigen to rabbits and induce a strong and lasting serum IgG response. However, it is not effective at inducing an IgA response. If this failure of the virus to induce a good IgA antibody response was due to the lack of Th2 cytokines, then co-expression of the foreign antigen with Th2 cytokines such IL-5 and IL-6 might enhance the IgA response. This is worth further investigation because it may have implications for developing immunocontraception targeted at sperm antigens.

6.3 Antigen presentation and immune response to ZPB, a self-antigen

6.3.1 Overcoming self-tolerance to ZPB

Female rabbits are normally immunologically tolerant to recombinant rabbit ZPB. This was demonstrated by a direct immunisation experiment where female rabbits needed 2-3 injections of ZPB protein in adjuvant to develop high titers of antibodies to

ZPB (Kerr *et al.* 1999). On the other hand, male rabbits, where ZPB is not a self-antigen, had a strong antibody response to ZPB following a single protein injection (Kerr *et al.* 1999). In contrast, infection of female rabbits with MV-ZPB induced antibodies to ZPB (Kerr *et al.* 1999; Chapter 5), which were similar in titer and kinetics to those of male rabbits infected with MV-ZPB (Kerr *et al.* 1999). The IgG was shown to bind to the ZP of rabbit oocytes and this was associated with follicle loss (Chapter 5). This raises the question of how the recombinant virus is overcoming immune tolerance in female rabbits.

One possible explanation is that tolerance is due to suppression or deletion of ZPB reactive T cells. However, if a viral protein-ZPB complex could bind to B cells through surface Ig specific for MV or ZPB, these cells could then present both ZPB and MV peptides on MHC-II molecules following internalisation and processing of the antigen complex. MV specific T cells, probably activated by APC cells, could activate these B cells. This model was proposed by Jackson *et al.* (1998) to explain how recombinant ectromelia virus expressing mouse ZPC could overcome immune tolerance and induce antibodies to ZPC. Another possible explanation is that viral infection creates an inflammatory micro-environment (enriched with proinflammatory molecules such as cytokines and other immune factors) which non-specifically increases the responsiveness of auto-reactive T and B cells to self-antigen.

Mouse ZPC delivered by a recombinant mouse poxvirus induced an antibody response to this antigen and infertility in female mice (Jackson *et al.* 1998). Similarly, recombinant murine cytomegalovirus expressing mouse ZPC is able to induce antibody responses and infertility in infected mice (Chambers et al., 1999). Recombinant vaccinia virus, which delivered a mouse tumour antigen, overcame self-tolerance and induced an autoimmune response that caused the destruction of tumour cells (Overwijk *et al.* 1999). Taken together, these data suggest that the immune response induced by a recombinant virus to a self-antigen may be quite different to that induced by conventional immunisation.

6.3.2 A possible model for the short-lived antibody response to ZPB induced when rabbits were immunised with MV-ZPB

Although self-antigens delivered by recombinant viruses could overcome tolerance as described above, some level of immune-tolerance to the antigen still existed. This was suggested by the observation that only a short-lived antibody response and a weak inflammatory response in the ovary were induced in female rabbits infected with MV-ZPB (Chapter 5). These responses led to significant losses of pre-ovulatory or tertiary follicles associated with a low and transient infertility in some rabbits. Both antibody levels and infertility were enhanced by boosts of recombinant ZPB protein (Kerr *et al.* 1999); boosting was also associated with a partial depletion of primordial and primary follicles (Chapter 5).

The relatively transient antibody response to ZPB induced after MV-ZPB infection compared with immunisation with MV-HA (Kerr *et al.* 1999; Chapter 5), is postulated to be due to the lack of T memory cells. It is known that activated T cells play important roles in B cell differentiation and antibody production (Roitt, 1997) for example through CD40 and CD40 ligand interaction (Kooten and Banchereau, 2000). Without T memory cells, the B cell response and antibody production may not be long-lasting (Roitt, 1997). If we postulate that the poorly sustained antibody response to ZPB is due to a lack of memory T cells, then this may reflect T cell tolerance to ZPB during CD4+ T cell activation. One explanation for this is that ZPB-reactive T cells are anergic through the mechanism of peripheral tolerance (Lo, 1992; Roitt, 1997). Another possibility, as suggested by de St Groth (1998), is that the lymphoid dendritic cells (LDC) could distinguish a self-antigen (ZPB) from a foreign antigen (MV) and so lead to self-tolerance. The finding that CD4+ T cells were crucial for the antibody response to a self-tumor antigen delivered by recombinant vaccinia virus (Overwijk *et al.* 1999) supports the view that Th memory cells are important to a sustained immune response to a self-antigen. Therefore, overcoming T cell tolerance by enhancing the immunogenicity of ZP antigens and antigen presentation to CD4+ T cells could be important for inducing a high and lasting antibody response to ZP antigens.

Another explanation for the short-lived antibody response to ZPB is that since the recombinant MV was rapidly cleared from the host, viral-ZPB antigen complexes or

ZPB protein were no longer available to bind to B cells. The B cells for ZPB antibodies then could not be activated by MV specific T cells, which would result in a short-lived antibody response. From this point of view, keeping the recombinant virus longer in the host, and thus a sustained production of ZPB, might lead to a sustained antibody response to ZPB. If ZPB is persisting but the immune response is not sustained, this would suggest that regulatory mechanisms are acting to suppress the autoimmune response and this contrasts strongly with the high titer and sustained antibody responses to HA. Further experiments are needed to determine whether tolerance is mediated at the level of B or T cells before a detailed explanation can be developed.

6.4 A hypothesis for the relationship between follicle losses and infertility induced by MV-ZPB infection

A reduction in the number of follicles in the ovary is considered to be one important factor contributing to infertility in rabbits immunised with ZP antigens (Skinner *et al.* 1984; Jones *et al.* 1992; Kerr *et al*, 1999). But significant reduction of only pre-ovulatory or tertiary follicles with retention of normal primary or primordial follicles produced low rates of infertility (Kerr *et al*, 1999, Chapter 5). However, significant reduction of primary or primordial follicles as well as pre-ovulatory or tertiary follicles may result in significant and sustained infertility. In addition, although IgG to ZPB was postulated to be the main factor responsible for follicle loss in rabbits (Chapter 5, Skinner *et al.* 1984), it did not seem to directly disrupt primary and primordial follicles. This was suggested by the observation that primary and primordial follicles remained basically normal at 15 and 30 days post-infection with MV-ZPB, whereas tertiary follicles were significantly disrupted in the same infected rabbits (Chapter 5). From the time course of ovarian pathology, it appeared that primary or primordial follicle reduction happened at a later stage of ovarian pathology and needed a persistent, high-titered antibody response (Chapter 5). Based on these observations, a two-stage model of ovarian pathology is postulated to explain the relationship between antibody response, follicle reduction, and infertility.

Stage 1 is dominated by IgG mediated tertiary or secondary follicle degeneration and disruption. During this stage, an antibody response to ZPB is mounted and IgG antibody binds to ZP of the oocytes in the ovary. It is also possible that the binding of IgG to ZP could prevent sperm from attaching to the oocytes following ovulation or inhibit correct development of the oocyte thus resulting in a fertilisation block (Chapter 1). In this model, the binding of antibody to the ZP would initiate ovarian pathology by causing tertiary or secondary follicle degeneration and disruption (Chapter 5). Because most primary and secondary follicles are unaffected at this stage (Chapter 5), the infertility rate may not be heavily influenced by ovarian pathology such as follicle losses and ovarian dysfunction but may be more influenced by failure of fertilisation following binding of antibody to the ZP. If the immune response ceased at this stage, follicle development and fertility may be easily recoverable.

Stage 2 is dominated by primary and primordial follicle disruption and ovarian dysfunction. As the disruption of tertiary or secondary follicles in stage 1 continues, the ovary develops severe pathology (such as accumulation and expansion of LPC), presumably accompanied by endocrine dysfunction. Most primary and primordial follicles are lost. This could be due to ovarian pathology and endocrine dysfunction that makes the transformation of primordial to primary follicles and the subsequent development of primary follicles impossible. Alternatively, primordial and primary follicles may be recruited into the developing follicle pool where they are disrupted by IgG antibody at such a rate that primordial follicle depletion was observed. This means that for sustained infertility long-lasting IgG antibody and continuous depletion of newly formed tertiary or secondary follicles would be important. Because of the sparcity or absence of tertiary or secondary follicles in the ovary in this stage, direct antibody blocking of fertilisation would be less important. This makes the ovarian and endocrine dysfunction that caused the ovary to fail to produce oocytes the main cause of infertility. This stage could take much longer to develop (6-7 weeks) according to the current results (Chapter 5) and previous studies with porcine ZP immunisations (Wood *et al.* 1981; Skinner *et al.* 1984; Jones *et al.* 1992). Recovery from this stage could be prolonged or impossible.

According to this model, IgG antibody and primordial and primary follicle atresia are both important for inducing a high infertility rate. A high titer of IgG antibody may be

more important for directly blocking fertilisation by binding to the ZP at stage 1 but follicle disruption may be more important for infertility during stage 2. A long-lasting antibody response is crucial for sustaining the ovarian pathology. MV-ZPB infection induces only a low and short-lived antibody response and ovarian pathology stayed at stage 1. This would explain the low infertility rates in female rabbits after MV-ZPB infection (Kerr et al. 1999). Boosting of the MV-ZPB infected rabbits with recombinant ZPB protein induced a sustained antibody response, led the ovary into stage 2 of the pathogenesis and resulted in the higher infertility rate in boosted rabbits (Kerr et al. 1999). Although the induction of infertility has been divided into two stages in this model, the two stages may not be critically divided. For example, loss of primary follicles was seen in some individuals in the day 15 and 30 groups of MV-ZPB infection (Chapter 5, Table 5-4). But, in general, the tertiary follicles were disrupted before primary or primordial follicle reduction at the level of antibody induced by MV-ZPB infection. Because T cells were only seen in ovaries from one rabbit at day 5 and one rabbit at day 15 following infection, the role of T cells in follicle disruption is unclear and has not been developed in this model.

6.5 Future studies

6.5.1 Enhancing immunogenicity of ZP to induce a stronger antibody response

MV-ZPB showed the potential to be an immunocontraceptive vaccine (Kerr et al, 1999; Chapter 5). However, tolerance needs to be more fully overcome to induce a stronger and lasting IgG antibody response to ZPB for a higher infertility rate. Further studies could include co-expressing adjuvant cytokines such as IL-2 with ZPB in the virus to enhance the T cell response to ZPB. As an alternative strategy, using heterologous ZP antigens such as porcine ZPC instead of rabbit ZPB to increase the immunogenicity of ZP antigens may overcome self-tolerance. In the monkey, immunisation with recombinant rabbit ZPA (75-Kd ZP) protein showed interference with ovarian follicular development and function whereas immunisation with rabbit ZPB protein showed an antibody response but no effects on follicular development and

ovarian function (VandeVoort *et al.* 1995). For the rabbit, selecting other ovarian antigens such as rabbit ZPA, ZPC or co-expressing these antigens with ZPB might be able to enhance the immunogenicity of ZP antigen and achieve a better sterilisation effect or fertilisation block. In addition, for the long-term, identifying other immunocontraceptive ovarian antigens such as antigens from granulosa cells would also be worthwhile.

6.5.2 Understanding LPC and their effect on follicle development and ovarian function

It was shown that LPC were associated with follicle loss and ovarian pathology (Chapter 5). Whether these LPC are the result of pathology or are capable of causing further ovarian dysfunction is wholly unknown. It would therefore be worthwhile to investigate how LPC are induced, what kind of hormones, if any, they produce, and which hormones, if any, regulate these cells. Measurement of oestradiol, progesterone, FSH and LH levels in serum may facilitate the understanding of ovarian dysfunction and the mechanisms underlying the infertility that follows ovarian pathology.

6.5.3 Passive immunisation experiments with anti-ZPB IgG

Comparison of MV-ZPB and MV-HA infections showed that infections with myxoma virus itself caused some follicle degeneration (Chapter 5). To investigate the role of IgG to ZPB in ovarian pathology, in the absence of the virus and to understand the role of IgG in the time course of ovarian pathology, an IgG antibody passive immunisation experiment would be necessary. Anti-ZPB IgG can be purified and injected into female rabbits. Considerable preliminary study would be needed to establish the injection does and regime necessary to maintain high serum antibody levels for the duration of the experiment. Immunological and histological changes in the ovary and infertility rates would then be monitored over a longer time period (at least 7 weeks) to observe the changes in tertiary (stage 1) and primary or primordial (stage 2) follicle numbers as well as other ovarian pathology, for example the development of LPC. This study is relatively simple in concept but purification of the large amounts of

ZPB antibodies needed and the development of an appropriate regime for their delivery would be demanding.

6.5.4 Further studies of mucosal immunity in the female reproductive tract

The presence of IgG and IgA in uterine epithelial cells showed that both antibodies are able to transfer from tissue to epithelial cells (Chapter 3 and 4). Detection of secretory component on epithelial cells or conducting a passive Ig transfer assay could provide more information about antibody transfer into these cells and to the mucus and lumen of the reproductive tract. IgG and IgA was highly concentrated within the cells of the epithelial surface of the reproductive tract, especially the uterus. This was shown both immunohistochemically and by the low levels of IgA in washings from the reproductive tract. Further characterisation of IgA isotypes and their potential functions is important for understanding mucosal antibody responses in the reproductive tract.

At the T cell level, investigating and characterising the IEL might enable an understanding of their functional roles. The number of KEN-5+ T cells, IL-2 mRNA expression and MHC-II+ cells increased dramatically in the uterus after ovulation. Understanding their relationship to mucosal immunity or reproduction will increase our knowledge of reproductive immunology of rabbits.

6.6 Conclusion

This study classified the basic immune cells in the female rabbit reproductive tract and investigated the immune response in the tract to a model antigen HA and an ovarian antigen ZPB delivered by recombinant myxoma viruses. The recombinant virus was an effective vector at inducing antibody responses within the ovary but not within the mucosal part of the reproductive tract for immunocontraception. MV-ZPB has the potential to be an immunocontraceptive vaccine but a high and lasting IgG response to ZPB is needed to achieve higher infertility rates.

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